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The role of Trans-sialidase on Trypanosoma cruzi parasite load in Rhodnius prolixus, impact of infection on triatomid behavior, and dispersal in a simulated field environment.

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THE ROLE OF *TRANS*-SIALIDASE ON *TRYPANOSOMA CRUZI* PARASITE LOAD IN *RHODNIUS PROLIXUS*, IMPACT OF INFECTION ON TRIATOMID BEHAVIOR, AND DISPERSAL IN A SIMULATED FIELD ENVIRONMENT

A Dissertation Presented

by

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To Sonny and my parents:

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ABSTRACT

THE ROLE OF *TRANS*-SIALIDASE ON *TRYPANOSOMA CRUZI* PARASITE LOAD IN *RHODNIUS PROLIXUS*, IMPACT OF INFECTION ON TRIATOMID BEHAVIOR, AND DISPERSAL IN A SIMULATED FIELD ENVIRONMENT

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Studies were undertaken to observe: (1) the role of *trans*-sialidase (TS) on *Trypanosoma cruzi* parasite load in *Rhodnius prolixus*, (2) behavioral implications of *T. cruzi* infection in *Rhodnius prolixus*, and (3) movement and attraction of laboratory *R. prolixus* within a simulated field environment.

Laboratory *R. prolixus* were infected with *T. cruzi* parasites (Silvio strain) with or without various TS types. The addition of *Trypanosoma* TS or any recombinant TS type did not consistently affect the parasite load. However, the addition of 1.0 μg TS monoclonal antibody/ml blood did significantly increase parasite load.

Fractionated *T. cruzi* subpopulations (Silvio strain), based on the phenotypic expression of TS, were fed to *R. prolixus*. Bugs ingesting parasites lacking TS expression (TS^- parasites) produced significantly higher parasite loads than bugs ingesting either TS^+ parasites (parasite phenotypically expressing TS) or unfractionated populations. Two other strains of *T. cruzi* (Tulahuen and Montalvania-13) were fractionated by TS phenotype and fed to bugs. Fractionated Tulahuen parasites only weakly produced

differential parasite loads, the highest of which was produced by TS^- parasites, but Montalvania-13 TS^- parasites produced significantly larger parasite loads than either TS^+ parasites or unfractionated parasites.

The feeding and defecation behavior of *T. cruzi* infected vs. uninfected insects were observed on an artificial membrane-feeding system, as well as on a live host (guinea pig) feeding system. Fifth instars were the best vectors, followed by adult females, 4th instars and finally, adult males. Bugs fasted for longer periods of time (5-6 months) took smaller blood meals but defecated significantly earlier than bugs fasted for shorter periods of time (2-3 months).

A field environment was simulated within a styrofoam box. Movement of *R. prolixus* into various refuge types was observed. Fifth instars were more likely to seek a refuge than 3rd instars. Fed nymphs were more likely to seek a refuge than fasted nymphs. Potential bug attractants were tested within an olfactometers; ammonium hydroxide (NH_4OH) was incorporated into refuges placed in the simulated field environment. Fasted 4th instars significantly preferred to rest in refuges containing NH_4OH whereas fed 3rd instars seemed to actively avoid refuges containing this chemical.

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CHAPTER 1

LITERATURE REVIEW

Chagas' disease

American trypanosomiasis, also known as Chagas' disease, afflicts an estimated 17 million people in Latin America (World Health Organization, 1997). The etiologic agent is *Trypanosoma cruzi*, a hemoflagellate protozoa (Kreier and Baker, 1987). Transmission can occur through a variety of routes: (1) contamination of a mucosal membrane or wound by infective *R. prolixus* feces, (2) ingestion of infected bugs or water or food contaminated by their feces, (3) congenital transmission, (4) blood transfusion, or (5) sexual transmission (Prata, 1994).

The disease manifests itself in two stages: the acute and the chronic phase. The acute phase includes such symptoms as: malaise, fever, aches, and lymphadenopathy (Kirchoff, 1993; Prata, 1994). Classic symptoms for the acute stage include a swelling of the region surrounding the eye (Romana's sign) or an edema at the site of a kissing bug bite, known as a chagoma (Harwood and James, 1979). Adenopathy and sometimes nervous disorders may also develop (Tanowitz et al., 1992). The acute phase is more common among small children, causing a mortality of 10-15% (Schofield, 1994). The acute phase usually lasts about 4 weeks, after which the patient recovers and an equilibrium is established between the host and parasite; this is referred to as the chronic phase of the disease. Blood parasitemia is extremely low, as the parasites enter various organs of the body. Some patients (10-30%) suffering from the chronic phase may

develop new manifestations of the disease 10-40 years after the original infection (Kirchoff, 1993; Schofield, 1994). The chronic phase is characterized by megasyndrome (enlargement of the heart, esophagus, spleen, or colon) and sudden death may occur at any time. Different parasite strains, geographically distinct, preferentially affect different target organs.

Currently, there is no vaccine available for the treatment of Chagas' disease. Although there are some forms of chemotherapy (e.g., nifurtomox, benznidazole) used to treat the acute phase of the disease, these chemicals have high mammalian toxicity: 30% of patients treated in this manner develop serious side effects such as nausea, vomiting, anorexia and sleeping disorders. There are no drug treatments available for the chronic phase; organ transplants are the only realistic option.

In general, sufferers of Chagas' disease are those of lower economic standing. Houses constructed with plant material or mud walls fail to provide protection against kissing bug infestations. The three most domesticated species of kissing bugs are *Triatoma infestans*, *Rhodnius prolixus,* and *T. dimidiata* (Zeledon, 1974). These species readily invade homes and spread to other areas through human transport, by hiding in boxes and firewood.

Chagas' disease is of great economic importance in Latin America, as people lose work days in the acute phase and suffer from debilitating ailments in the chronic stage. In the worst case scenario, people suddenly die from a heart attack not even knowing they have the disease. Treatment options are expensive: drugs, insecticides for vector control, and medical treatment all require significant amounts of money. Afflicted poor people usually either cannot afford early diagnosis or treatment.

Trypanosoma cruzi

There are three basic morphological forms of *T. cruzi*: trypomastigotes, epimastigotes and amastigotes. Trypomastigote forms are found both in the extracellular system of the insect (metacyclic trypomastigotes), as well as in the mammals (bloodstream trypomastigotes). Trypomastigotes are characterized by their long, undulating flagella, which extends along most of their bodies, causing the protozoa to have a whip-like movement. Separation of trypomastigotes from epimastigotes is based on the location of the kinetoplast in relation to the nucleus. In trypomastigotes, the kinetoplast is located at the very posterior of the organism, quite removed from the nucleus; the kinetoplast and nucleus are contiguous and/or indistinguishable from one another in the epimastigote form. Epimastigotes are found only in the insect and replicate via binary fission in the insect's midgut. Amastigotes are intracellular parasites in mammalian tissue.

Insect trypomastigote forms are much more infective to vertebrates than bloodstream trypomastigotes (Villalta and Kierszenbaum, 1987). Also, the addition of *R. prolixus* extracts to cultured *T. cruzi* parasites significantly increased the production of metacyclic trypomastigote forms, suggesting that insect forms of the parasites are well-adapted to survive in the digestive system of the bug (Wood and Sousa, 1976) and to infect the mammalian system.

The life cycle of *T. cruzi* alternates between a mammal and a triatomine insect. Uninfected insects ingest bloodstream trypomastigotes when feeding upon an infected mammal; within a few days the trypomastigotes transform into epimastigotes in the insect midgut and travel to the rectum, replicating by binary fission along the way.

Epimastigotes attach themselves at the rectal gland and transform into metacyclic trypomastigotes and eventually, are passed with the feces. After an extrinsic incubation period of approximately 30 days, infective metacyclic trypomastigotes can be detected in the insect feces. When the infective kissing bug is ready to take another blood meal, it can infect a naive human being or mammal by depositing its infective feces near the bite site or mucosal membranes such as the eye. As host irritation occurs, the feces may be scratched into the skin or eye and infection occurs. After an intrinsic incubation period of 4-11 days, the acute phase of Chagas' disease begins as the trypomastigotes enter the bloodstream, invade a variety of host cells and transform into amastigotes which replicate through binary fission. Amastigotes transform into trypomastigotes, which are released into the bloodstream as the infected cell eventually ruptures, and become available to be picked up by another triatomine vector. This cycle of transmission can occur over and over again unless the chain is broken either through vector control or treatment of infected persons with therapeutic drugs.

Host-parasite interactions

Extracellular parasites residing in the mammalian bloodstream are vulnerable to attack by the host immune system and its arsenal of weapons. Hence, such parasites have an ongoing "arms race" with their host's immune system. Mechanisms must be continually evolved with which to avoid destruction. In turn, the host's defensive system must develop mechanisms to combat these parasite responses. The most successful parasites are those which are able to develop long-term infection of the host, characterized by low parasitemia. Such chronic infections represent a type of homeostasis, where the

parasites continue to persist and multiply in the host body, but the host system has been able to control and maintain parasitemia at tolerably low levels.

Since the 1970's, researchers have known that trypomastigote forms of *T. cruzi* are extremely susceptible to lysis by the complement system in refractory animals, such as birds and amphibians (Kierszenbaum et al., 1976). Subsequent research in the 1980's and 1990's has provided evidence that survival of the parasite is distinctly stage-specific, or developmentally-regulated. Evidence of adaptation for survival in the mammalian bloodstream environment include: (1) epimastigote (insect forms) are extremely susceptible to lysis by complement, (2) trypomastigote forms have developed resistance mechanisms to avoid cellular lysis by inhibiting the steps of the complement activation pathway, and (3) amastigote (intracellular) forms do not inhibit complement pathway activation (Schenkman et al., 1994). Epimastigote forms are non-infective for humans and thus, it is not surprising that they exist only as the replicative machinery in the invertebrate host. Trypomastigotes are constantly under attack from the host's immune system and have therefore evolved mechanisms to resist lysis by the complement system. Intracellular amastigote forms do not need to protect themselves against complement factors since they never enter the extracellular environment and are already protected within the cell.

The direction of future research points towards targeting genetic sites of the parasite for manipulation of the various proteins regulating complement resistance. This information could then be applied to current vaccine development or attempting parasitological cures in both the acute and chronic phases of Chagas' disease. The discovery of other proteins and their involvement in complement resistance seems

inevitable and necessary to fully understand these mechanisms in order to deactivate them and purge the parasites from the mammalian system.

Trans-sialidase (TS)

Since its recent discovery, researchers have been investigating the role that *Trypanosoma trans*-sialidase plays in Chagas' disease transmission and development. *Trypanosoma trans*-sialidase enzymes are of biological significance because they catalyze two reactions: (1) sialidase or neuraminidase activity which releases sialic acid to the environment (Pereira, 1983) and (2) transferase activity in which α(2,3)-linkages of sialic acid are transferred to appropriate beta–galactose acceptors (Schenkman et al., 1991). The end products of these reactions are sialyloglyconjugates. Sialic acids transferred from host glycoconjugates to acceptor molecules of the plasma membrane of a pathogenic microbe form an epitope known as Ssp-3. This trypomastigote-specific epitope is suspected of having target recognition properties because monoclonal antibodies which recognize Ssp-3 block the attachment of trypomastigotes to the host cell membrane (Schenkman et al., 1991). In addition, sialic acids are critically involved in cellular interactions and the mediation of pathogenic microbe recognition by mammalian host cells (Schenkman et al., 1994).

Trans-sialidase (TS) is a developmentally-regulated enzyme, produced in the highest levels by the trypomastigote and 10-fold less by the epimastigote form of *T. cruzi* (Pereira, 1983). Epimastigote TS, in addition to being developmentally-regulated and produced in increasing amounts as the stationary phase is reached, also significantly differs

from trypomastigote TS by lacking the carboxyl-terminus tandem repeats (Chaves et al., 1993; Briones et al., 1995). Amastigotes do not express TS (Pereira, 1983).

Sialylation of the trypomastigotes has been demonstrated to provide some degree of protection from lysis by host complement components (Tomlinson et al., 1994). Kierszenbaum and Ramirez (1990) demonstrated that those parasites capable of resisting complement did not represent a genetically-resistant parasite population; a certain proportion of trypomastigotes were lysed each time, regardless of successive culturing of resistant parasites. *Trans*-sialidase has also been implicated as having a role in the invasion of mammalian cells and enhancing parasite virulence (Chuenkova and Pereira, 1995).

The first sialic acid of *T. cruzi* was discovered in 1980 and *T. cruzi* sialidase (=neuraminidase) was identified 3 years later. *Trans*-sialidase activity of this neuraminidase was discovered in 1991 by Schenkman et al. (1991). When scientists realized that both the neuraminidase and *trans*-sialidase activities originated from the same gene, they discovered that *trans*-sialidase, in the absence of a suitable acceptor, would have neuraminidase activity (Uemura et al., 1992). As the number of sialic acid acceptors increases (due to transferase activity), the amount of free sialic acid acceptors decreases (neuraminidase activity). Consequently, one enzyme alone could theoretically drive the reaction in the direction of either neuraminidase or transferase activity; the direction of the reaction would be dependent upon the concentration of available sialic acid receptors.

The primary sequence of TS has been divided into 4 theoretical domains (Schenkman et al., 1994). These regions are (from the amino terminus to the carboxyl-terminus): (1) a cysteine-rich region (457 amino acids); (2) type III module (135 amino

acids); (3) long terminal tandem repeat (535 amino acids) and (4) a glycosyl phosphatidylinositol anchor (GPI).

The structural form of TS (pH optimum = 6.0 - 6.5; pI = 6.5) is dependent upon the developmental stage of the parasite (Pereira, 1983). The relative sialidase activity of epimastigotes is 1, while that of metacyclic trypomastigotes is 10 - 20 fold higher and that of bloodstream trypomastigotes is $\geq$ 20-fold higher (Pereira, 1983; Harth et al., 1987; Cross and Takle, 1993). There is no measurable sialidase activity in amastigotes. The TS of epimastigotes is monomeric while that of trypomastigotes consists of multiple subunits and contains an additional domain (12 amino acids, active in oligomerization) at the carboxyl-terminus (Chaves et al., 1993; Briones et al., 1995). Genetic studies have illustrated that native trypomastigote TS is composed of two separate domains: (a) a conserved region, located at the amino-terminus, which contains the active site and (b) a heterogenous region of tandem repeats, located in the carboxyl-terminus, which contains a domain critical for enzyme oligomerization or aggregation (Schenkman et al., 1994). The latter site is highly antigenic in the mammalian system; its presence has also been discovered to increase the immune response to the catalytic domain, which then acts to inhibit *trans*-sialidase activity (Buscaglia et al., 1998).

Trypomastigote TS is anchored to the membrane of the parasite by a glycosyl phosphatidylinositol anchor (GPI) while the epimastigote TS is attached by a normal trans-membrane domain (Schenkman et al., 1994). Epimastigote TS is not released from the parasite membrane surface, even when exogenous phospholipase is added. However, evidence indicates although the two TS enzymes of the epimastigote and trypomastigote forms are different, their catalytic domains are structurally related (Chaves et al., 1993).

TS may be involved in the manifestation of Chagas' disease pathology. Antibody studies (for both the acute and chronic phases) indicate that immune responses to the parasite are targeted towards specific domains of TS; antibodies isolated from sera of chronic human sufferers and from mice and humans with acute infections inhibit TS activity (Leguizamon et al., 1994a, 1994b; Pereira-Chioccola et al., 1994). Also, Pereira and Hoff (1986) discovered that *T. cruzi* strains, which were histotropic for macrophages or cardiac muscle cells, have lower neuraminidase activity than strains which are histotropic for skeletal muscle.

Trypanosoma cruzi trypomastigote populations are apparently not homogeneous. Though the existence of two trypomastigotes morphologies has been observed for decades, researchers were unable to work with purified parasite subpopulations and were forced to work with strains predominated by either broad or slender forms. Brener (1971) noted that different strains had different proportions of each morphology and surmised that slender forms were more suited towards invading the cells of mammals than the broad forms and that only broad forms were cycled through the insect.

Through the use of a monoclonal antibody (TCN-2) binding to the carboxyl-terminus tandem repeats of TS, Pereira et al. (1996) discovered that only a small subset (20 - 30%) of *T. cruzi* trypomastigotes (Silvio strain) produce TS (TS^+); these trypanosomes were more invasive than those not producing TS (TS^-). The addition of exogenous TS to TS^- parasites transformed them into the invasive phenotype, indistinguishable from TS^+ parasites. In the microscopic examination of the Silvio strain of *T. cruzi*, TS^+ parasites are the broad forms and move very slowly, while the TS^- parasites move with quick, whip-like movements and are very slender.

However, each *T. cruzi* strain differs in its proportion of TS phenotypic expressive parasites and the morphology differs from strain to strain. *Trypanosoma cruzi* strains also differ in their overall production of neuraminidase, which may or may not be directly correlated with the proportion of parasite phenotypically expressing TS (Pereira and Hoff, 1986).

The biological role and significance of TS interaction with *T. cruzi* parasite load in the kissing bug vector is unknown. Amino et al. (1995) found that parasites recovered from gut homogenates of *Triatoma infestans* were poorly sialylated and attributed it to a sialidase activity native to the bug gut; they suggested that insect forms of *T. cruzi* did not require sialic acid in order to successfully survive in the insect gut. Alvarenga and Brener (1978) fed a "blood-free" diet to *T. infestans* and *Dipetalogaster maximus* and discovered that *T. cruzi* did not need a blood meal in order to survive and develop in the bug. It has also been hypothesized that sialylation of the *T. cruzi* parasite may enhance its survival within the insect vector, where sources of sialylated carbohydrates could only be provided through the blood meal or intestinal flora (symbiotic bacteria). Perhaps the sialylation protects the parasite from enzymatic action within the vectors themselves, affects its adhesion to the insect's gut wall, or protects it from the actions of host complement (Schenkman and Eichinger, 1993).

Trans-sialidases have been identified also in other pathogenic microbes such as African trypanosomes, *Endotrypanum* sp., and *Pneumocytis carini* (Schenkman et al., 1994). However, their role in these organisms has not yet been elucidated. In *Trypanosoma brucei*, only the insect forms of the parasite express *trans*-sialidase; procyclin is the only molecule sialylated on the parasite surface, in addition to its GPI

anchor (Pontes de Carvalho et al., 1993). These researchers hypothesized that the sialylation of procyclin may affect the parasite's ability to adhere to the peritrophic membrane or epithelial cells in its eventual migration to the salivary glands or, may provide protection against residual activity of the host complement system remaining with the ingested blood meal.

Rhodnius prolixus (Reduviidae: Hemiptera)

The family Reduviidae contains 118 species of Triatominae bugs (Schofield, 1994). Though all are blood-sucking and more than half of these are capable of transmitting *T. cruzi*, only about a dozen species vector the parasite consistently and are considered important natural vectors (Harwood and James, 1979). Of these twelve, there are seven primary vectors of Chagas' disease in Latin America, confined to three genera: *Triatoma infestans, T. dimidiata, T. sordida, T. brasiliensis, Rhodnius prolixus, R. pallescens,* and *Panstrongylus megistus* (Zeledon, 1981; Schofield, 1994). All of these bugs are domesticated, living in very close association with man, which behaviorally pre-disposes them as effective transmitters of this disease to humans.

Rhodnius prolixus is an important vector in Colombia, Venezuela and certain non-contiguous parts of Central America (Ruiz, 1953; Schofield, 1994; Dujardin et al., 1998). There are two hypotheses to explain the isolated presence of *R. prolixus* in Central America: (1) migrating storks carried nymphs and eggs with them and (2) they are the descendants of laboratory escapees (Dujardin et al., 1998). They can be discriminated from members of the genus *Triatoma*, based on the location of the antennae relative to the

beak and the eyes: in *Rhodnius* spp., the antennae are located very anteriorly along the snout, whereas in *Triatoma* spp., the antennae are in close proximity to the eye.

The physiology and life history of *R. prolixus* was studied extensively during the first half of the 20th century, due to the ease of their laboratory maintenance and reasonably large size. These early studies gave detailed information regarding the feeding frequency and molting activity of the five nymphal instars, as well as preliminary reports on the autogeny and fecundity of adults (Uribe, 1927; Buxton, 1930; Wigglesworth 1931a, 1931b, 1931c, 1934; Goodchild, 1955). However, until the advent of J. C. Gomez-Nunez in the 1960's and C. J. Schofield in the 1970's, behavioral studies of triatomines remained relatively nonexistent.

Rhodnius prolixus populations are found both in the sylvatic and peridomestic environments. Although *R. prolixus* is extremely well-adapted to life within human dwellings, wild populations can be found in or near animal nests among palm fronds. Some dispersal of *R. prolixus* is attributed to bird migration, though most is probably executed by human movement and the transport of various goods. Such action also helps to regulate bug populations (Rodriguez and Rabinovich, 1980) as does host availability (Gurtler et al., 1992). Populations with higher densities, though experiencing prolonged developmental times, do not have lower survivorship (Rodriguez and Rabinovich, 1980).

Food source orientation

Several studies have determined that fifth instar *R. prolixus* nymphs are attracted to components such as heat, host odor, carbon dioxide and moisture (Friend and Smith, 1977; Schofield, 1977; Nunez, 1982). *Rhodnius prolixus* will probe at warmed surfaces

and sample warmed fluids lying beneath a membrane (Friend, 1965). However, the most attractive stimulus is a natural host, such as a hamster or human. Minter (1976) suggested that the proximity of the host is an important factor in host selection.

Gomes (1990) tested various host blood sources and determined that the life history parameters of *R. prolixus* were optimal when the insects were fed either human or rabbit blood. Usually one full blood meal is sufficient for the insect to molt successfully to the next life stage, but an extra blood meal is often required for the fifth instar (Goodchild, 1955). Compared to *T. infestans*, *R. prolixus* is a more effective bloodsucker; the relative blood meal capacity to insect body weight of *R. prolixus* is much greater than that of *T. infestans* (Pereira et al., 1998). However, the host irritation caused by *T. infestans* was significantly less than that of *R. prolixus*, suggesting that it is easier for *T. infestans* to take smaller but more frequent blood meals.

Most kissing bugs are opportunistic feeders and can feed on a wide variety of hosts. *Rhodnius prolixus* will feed on: rodents, poultry, wild birds, dogs, cats, bats, opossums, lizards, man, and sometimes other bugs (Carcavallo, 1987). The general types of ecological habitat that *R. prolixus* has been found are: subtropical forests, dry and subtropical forests and xerophytic forests (Carcavallo, 1987). *Rhodnius prolixus* is considered to be a major vector in Venezuela, Colombia, in parts of Central America such as Guatemala, Honduras, and El Salvador, where the insect vector is found primarily in the domestic environment (Schofield, 1994). In Venezuela and Colombia, *R. prolixus* is found in the domestic environment and sylvatic habitat, but in Central America, this bug vector is found primarily in human dwellings. The presence of *R. prolixus* has also been recorded in other parts of Latin America, but these populations are not considered to be

major vectors, probably because their habitats have limited or reduced contact with humans.

Pheromones and attractants

In addition to producing isobutyric acid as an alarm pheromone (Schofield, 1994), *R. prolixus* also produces a sex pheromone. An airstream passed over a mating pair of bugs appears to sexually stimulate uncoupled males (Baldwin et al., 1971). Schofield and Patterson (1977) suggested the existence of a pheromone in the fresh feces of *R. prolixus* and *T. infestans*, which served to attract unfed nymphs and arrest the movement of recently fed nymphs. Further work with *T. infestans* suggested that these insects use their feces to mark their refuge with an aggregation pheromone (Figueiras et al., 1994; Lorenzo and Lazzari, 1996).

A few studies have investigated whether or not a chemical cue exists which could attract kissing bugs and be employed for use within a trapping device for either detection or control measures. Taneja and Guerin (1997) found evidence that low concentrations of ammonia were attractive to *T. infestans* nymphs. Similarly, Guerenstein et al. (1995) demonstrated within their laboratory that the production of carbon dioxide by yeast (*Saccharomyces cerevisiae*) attracted *T. infestans* nymphs.

Dispersal and resting place preferences

There have been few studies on the dispersal behavior of *R. prolixus* within the peridomestic environment. Rabinovich et al. (1979) found that 50% of the *R. prolixus* population was found along the bedroom walls. Gomez-Nunez (1969) followed a wild

population of *R. prolixus*, which was tagged with ^{60}Co. Preferred indoor resting places of wild *R. prolixus* included thatched roofs, household articles such as clothing and boxes, and wall-related refuges such as crevices or articles hung on the wall (Gomez-Nunez 1969). Gomez-Nunez (1969) reported that indoor *R. prolixus* were concentrated near areas where humans or other animals slept. Those insects released outdoors preferred to rest at the base of palm fronds. The outdoor dispersal range did not exceed 15 m while the indoor dispersal range did not exceed 4 m; these ranges were observed over a period of 40 days. Predation of *R. prolixus* in the sylvatic and peridomestic habitat was primarily by birds (24-64%), followed by rodents or reptiles (12-18%) and various arthropods (8-9%). Hunger was determined to be a motivational factor for *R. prolixus* dispersal and the local proximity to a host was a determining factor in refuge selection. In addition, the fasting level of a kissing bug may affect its resting place selection (Torres-Estrada and Ibarra, 1993, 1998). Torres-Estrada and Martinez-Ibarra (1993) discovered that *T. gerstaeckeri* nymphs preferred block walls if fed and mouse shelters if they were fasted. *Rhodnius prolixus* may also exhibit distinctive substrate preferences for oviposition (Schilman et al., 1996). Dispersal of *R. prolixus* may depend on such factors as: house construction materials, age of dwelling, and the availability of specific hosts (Zeledon and Rabinovich, 1981).

Vector competence

Within the medical entomology literature, there are several examples in which a parasite manipulates the physiology or behavior of its insect host to increase its chances of transmission. The first such example was the plague bacillus forming a plug impedes flea

feeding and causes the insect to regurgitate the bacilli in its repeated futile attempts to get blood (Bacot and Martin, 1914; Bacot, 1915). *Leishmania*-infected sand flies also suffer from the inability to feed normally; infected flies are unable to engorge normally. They repeatedly attempt to feed, obtaining only small blood meals, and increasing their chances of transmitting the parasite (Beach et al., 1985; Molyneux and Jefferies, 1985). Similarly, infected tsetse flies probe more frequently (Jenni et al., 1980), as did *Plasmodium gallinaceum*-infected *Aedes aegypti* (Rossignol et al., 1984) and *Plasmodium*-infected *Anopheles* mosquitoes (Morell, 1997). On the opposite extreme, Rossignol et al. (1985) discovered that mosquitoes feeding on animals infected with malaria or Rift Valley fever virus needed less time to find blood than those mosquitoes feeding on uninfected animals. Similarly, Day and Edman (1983) found that host defensive behavior is altered during malaria infection, enhancing transmission of the parasites to other uninfected mosquitoes. Coleman and Edman (1988) found that sandflies prefentially feed on sites of leishmania infection on mice.

The three difficulties that the parasite must overcome are directly related to vector competence: (1) finding a new host, (2) gaining entry into hosts, and (3) exiting from hosts (Baker, 1977). *Trypanosoma cruzi* finds a new host either when it is picked up from the bloodstream of an infected mammal by a kissing bug, or when it is deposited onto the skin or mucosal membrane within the bug's feces. The parasite gains entry into the insect host by direct ingestion and into the mammal by active movement. Departure from the host occurs via voided feces or by blood uptake by an insect.

Various aspects of behavior are also naturally selected to promote future transmission of insect genes or the parasites they carry. For instance, the faster an insect

feeds, the less chance it faces of being crushed while feeding (Gillett, 1966). Insects whose feeding rituals are painless have increased chances of survival by escaping detection by the host and decreasing host irritation.

There are several aspects of triatomine behavior that affect a given species ability to transmit *T. cruzi*: (1) degree of relationship with humans, (2) host preferences, (3) dispersion dependent upon climatic variables, (4) aggressiveness, (5) preferred resting places within the house, (6) densities of insects, and (7) the time when infective feces are deposited with regards to the completion of feeding (Zeledon, 1976). The degree to which a species has developed a relationship with humans is a reflection of the evolutionary path (Zeledon, 1976). The oldest human relationships are probably those represented by species such as *T. infestans* and *R. prolixus*, which are found predominantly in the peridomestic environment and much more rarely in sylvatic habitats. Other kissing bug species, not as well-adapted to the peridomestic habitat, are assumed to be in the process of evolving towards such a relationship because human populations are increasing and sylvan hosts are often declining.

Gurtler et al. (1992) found that the population density of *T. infestans* was dependent upon several factors: (1) history of pesticide use, (2) roofing material; (3) number of dogs sleeping in the same room as people ("roommate dogs"), and (4) number of human residents and "roommate" dogs of the house. Other factors which predispose *R. prolixus* as one of the most effective vectors of Chagas' disease are its infamous aggression, preference for thatched roofs, ability to attain high densities within human dwellings, and a quick defecation time with regards to the completion of feeding (Schofield, 1977; Zeledon, 1977; Rodriguez, 1980; Kirk and Schofield, 1987; Schofield,

1994). Rabinovich et al. (1979) found that the later instars were more likely to procure enough blood in one blood meal to successfully molt to the next life stage.

Wood (1951) did not observe any tendencies for kissing bugs to feed on particular parts of the animal, with or without regard to the presence of hair.

Defecation studies

Defecation patterns within a species of bug are dependent upon several variables: sex, instar and blood meal size (Piesman and Sherlock, 1983). Zeledon et al. (1977) compared the defecation rates of three major vectors of Chagas' disease and found that among *R. prolixus*, *T. infestans*, and *T. dimidiata*, *R. prolixus* defecated the earliest and in the greatest proportion. The following defecation index was proposed by these authors, to compare defecation patterns between various kissing bug species (Zeledon et al., 1977):

$$\frac{\text{\% defecating within 10 min} \quad \text{x} \quad \text{average no. of defecations in 10 min}}{100}$$

Trumper and Gorla (1991) timed the defecation of *T. infestans* and found that it was inversely proportional to the blood meal size. The researchers theorized that since smaller blood meals resulted during times of high population densities, that transmission of *T. cruzi* would be lower during these periods because host irritation levels would be increased. These levels of decreased feeding would prolong nymphal development, reduce female fecundity and longevity, and increase bug flight tendencies (Schofield, 1985). Kirk

and Schofield (1987) found similar results with *R. prolixus*, in that defecation occurred later with smaller blood meals.

The number of parasites present in the defecation of *R. prolixus* appeared to be dependent upon the number of parasites present in the previous blood meal and the amount of time that has elapsed since their last blood meal (Fistein and Chowdbury, 1970). However, the minimum number of parasites required to infect *R. prolixus* was found to be 177; infection with 1×10^3-1×10^4 produced the highest parasite loads (Neal and Miles, 1977).

Schofield et al. (1986) demonstrated that host perception of bug biting activity increased as bug densities increased, suggesting that host irritation serves to regulate bug feeding and population densities. Increased host grooming (to counteract biting irritation) in relation to insect feeding density also has been demonstrated in mosquito-host systems (Edman and Kale, 1971; Edman et al., 1972; Klowden and Lea, 1979; Waage and Nondo, 1982).

Catala (1994) criticized many laboratory experiments using starved insects fed through an artificial membrane or on a restrained host. He contends that, in nature, bugs are unlikely to obtain such large blood meals. Catala (1991) found that *T. infestans* attempts to feed frequently during the summer, but rarely during the winter. Though bugs are generally presumed to be infected for life, prolonged starvation appears to result in spontaneous abortion of *T. cruzi* infection in some insects (Vargas and Zeledon, 1985).

Trypanosoma rangeli infection

In addition to *Trypanosoma cruzi*, *R. prolixus* can be infected with *T. rangeli*. Unlike *T. cruzi*, *T. rangeli* is non-pathogenic towards man but displays pathogenicity towards *R. prolixus* (Grewal, 1957; Anez and East, 1984). *Trypanosoma rangeli* infection develops in the gut, hemolymph, and salivary glands of *R. prolixus*; only when invasion of the salivary glands has occurred has the cycle of development been successfully completed (Tobie, 1961). It is believed that *T. rangeli* is currently evolving from being a sterocorarian parasite (transmitted through posterior station transmission) to a salivarian parasite (transmitted through the bite) (Hoare, 1972). Insects infected with *T. rangeli* are unable to molt and exhibit higher rates of mortality, feed less frequently and probe blood sources more frequently (Grewal, 1957; D'Alessandro and Mandel, 1969; Anez and East, 1984). *Trypanosoma cruzi* does not appear to be pathogenic towards its insect hosts ((Schaub, 1988 and 1992; Schaub and Losch, 1989; Lima et al., 1992). However, Lima et al. (1992) did note that uninfected *Panstrongylus megistus* had significantly higher reproduction than infected insects; *T. cruzi*-infected *R. prolixus* have been observed to require slightly longer periods of time to reach ecdysis, though the difference was not significant (Takano, unpublished data).

Methods of Chagas' disease control and bug surveillance

Treatment options are expensive: (1) drugs, (2) insecticides, or (3) organ transplants (Marsden, 1988). Some researchers in the U.S. are attempting to transgenically manipulate the gut symbiont (*Rhodoccocus rhodnii*); transgenic symbionts are able to reduce or eradicate *T. cruzi* populations within the gut of *R. prolixus*

(Beard et al., 1992a, 1992b, 1993; Durvasula et al., 1997). However, vector control holds the most potential to reduce the incidence of Chagas' disease. Control methods are based on the ability to detect bug infestations, to quickly treat them with an insecticide such as a synthetic pyrethroid (Schofield, 1994) and to carefully monitor the house to ensure that the dwelling remains kissing bug-free.

Various methods of detecting kissing bug infestations in houses are thick or thin sheets of paper tacked to the wall (Garcia Zapata et al., 1985), sampling boxes filled with paper and tacked to the wall (Gomez-Nunez, 1965; Wisnivesky-Colli et al., 1987), or man-hours spent searching for insects (Pinchin et al., 1981; Gurtler et al., 1992, 1993; Rabinovich et al., 1995). Signs of an active infestation are considered to be exuviae, eggs, or live insect specimens.

CHAPTER II

MAINTENANCE OF THE *RHODNIUS PROLIXUS* COLONY

History of artificial maintenance

The artificial maintenance of arthropods has been occurring in the laboratory since 1912 or 1917, with the first non-live host feeding of tsetse flies (Tarshis, 1958). Artificial techniques have been employed for soft ticks (Kirch et al., 1991; Schwan et al., 1991) and hard ticks (Walaade et al., 1991; Walaade et al., 1996), mites (Bruce et al., 1991), fleas (Galun, 1966), lice (Mumcuoglu and Galun, 1987), bugs (Friend and Cartwright, 1963; Gardiner and Maddrell, 1972; Langley and Pimley, 1978; Gomes et al., 1990; Nunez and Lazzari, 1990; Cunha and MacCord, 1992; Pinto et al., 1992; Guerenstein and Nunez, 1994; Huebner et al., 1994), and flies such as mosquitoes (Bunner et al., 1989; Albuquerque et al., 1992), tsetse flies (Mews et al., 1976, 1977; Bauer and Aigner, 1978; Wetzel and Luger, 1978), biting midges (Jones, 1960; Hunt and McKinnon, 1990; Blackwell et al., 1994), black flies (Sutcliffe et al., 1979), and sand flies (Ghosh, 1994). Each different species comes with its own set of special requirements and there are usually several approaches to solve a problem.

There are many advantages to the artificial maintenance of arthropods in the laboratory. It allows the cost-effective large-scale production of arthropods, reducing animal rights issues and avoiding problems, which occur when hosts sometimes develop immune responses that reduce their effectiveness as a host. Artificial feeding systems also allow an increase in the number of studies on the transmission of bacterial, viral,

protozoan (Ghosh, 1994), and nematode (Albuquerque et al., 1992) pathogens by providing more controlled experimental conditions, eliminating concerns of insect density impact and leads to the permissibility of studies focusing on insecticides (Mullens, 1993; Hollbrook and Mullens, 1994).

However, there are several disadvantages with artificial feeding, as well. Unfortunately, a system without a live host fails to exactly replicate realistic circumstances present in natural blood feeding conditions and may have unforetold effects on the feeding organism, such as a resulting increase in mortality, decrease of fecundity (Bunner et al., 1988), altered development times and altered offspring size. There are problems with the blood source utilized; the blood is now more prone to contamination, it has a shelf-life to consider and its withdrawal is limited to the physiological health of the animal. Finally, experimental differences may be created, as was found to be the case with artificial vs. natural infection of *Culex tarsalis* mosquitoes with Western Equine Encephalitis (WEE) virus: under natural conditions, the virus was concentrated at the midgut periphery whereas under artificial infection conditions, the virus was more concentrated in the thoracic alimentary tract (Weaver et al., 1993).

There have been a limited number of blood administration systems developed so far, which can fit into broad categories: heat-sealed envelopes/tied condoms (Huebner et al., 1994); water-jacketed glass feeders covered with membrane (Garcia et al., 1975; Takano et al., unpublished results); membranes with blood above a heated structure (Mews et al., 1977); tubes covered with a membrane and submerged in solution (Mumcuoglu and Galun, 1987; Walaade, 1991, 1996), hanging drops, soaked pledgets, or egg shell membranes. A wider variety of membranes are utilized within these systems and

can be categorized as either natural or synthetic. Natural membranes are of animal-origin and consist of either animal skin (chick, mice, quail, even human) or their intestines (sheep or porcine). Artificial membranes can be composed of either silicon (Bauer and Wetzel, 1976), agar, parafilm, Baudruche, gutta percha, collagen (Cosgrove et al., 1994), or latex. Different orders and species of arthropods have different penetration abilities; one cannot generalize from one species to another, that one membrane will work across an entire order of organisms. Other factors to consider are membrane thickness, permeability, cost, reusability, and bacterial control.

In addition, phagostimulants can added or manipulated, to enhance feeding. Examples would be controlling the blood temperature or adding nucleotides such as ATP (Galun, 1966, 1967; Sutcliffe and McIver, 1979; Friend and Smith, 1982). There are other factors affecting the feeding success of insects through an artificial system: the hunger status of the insect, its physiological status with regards to egg development, and its age. The condition of the blood itself may affect arthropod feeding; the blood must contain the right concentration of phagostimulants, be of an appropriate viscosity and contain the proper blood fractions (Smith, 1978; Galun et al., 1985; Mumcuoglu and Galun, 1987). Methods of blood preservation also may have consequences on the insect (Wetzel, 1980); anticoagulants such as citrate, ethylenediaminetetraacetic acid (EDTA) or heparin may be used or blood fractions may be separated, frozen or freeze-dried.

Medical Entomology Laboratory *Rhodnius prolixus* colony

Members of the Medical Entomology Laboratory *R. prolixus* colony originated from Dr. Jose C. Ribeiro, who brought them from the Eloi S. Garcia research laboratory in Brazil. Dr. Garcia, in turn, brought the bugs from Costa Rica. Therefore, though our *R. prolixus* colony has been maintained since June 1995, they have been laboratory-raised since the early 1970's.

Rhodnius prolixus bugs were separated by instars in large glass jars, plentifully supplied by folded pieces of P4 filter paper. The wide-mouthed jars were covered with nylon, to permit bug feeding but prevent escape. Jars of bugs were maintained in the laboratory at 28° C and 70-80% relative humidity at a 14L:10D photoregime. Bugs were fed citrated rabbit blood, since it was demonstrated by Gomes et al. (1990) that *R. prolixus* developed optimally with either rabbit or human blood. Vacutainers containing sodium citrate (129 mM) were filled with 4.5 ml (vacuum-pressure) by Millbrook Farm, a local vendor. Blood was typically drawn the day before delivery and maintained thereafter in the refrigerator.

In our laboratory, the *R. prolixus* colony was maintained through an artificial membrane feeding system (Huebner et al., 1994) (Fig. 1). Individual parafilm envelopes were created by stretching two squares over the bottom piece (4 squares) and sealing the edges with a low-temperature glue gun. After the addition of citrated blood (ca. 10.0 ml), the remaining edge was heat-sealed.

The entire envelope was placed on a slide warmer, which was maintained between 36-40° C. Previous laboratory studies indicated that *R. prolixus* will feed on blood meals at various temperatures ranging from room temperature to 36° C (Pinto et al., 1992). Jars

were placed upside down and the insects allowed to feed until the envelopes were emptied of blood. The life history of these bugs is found in Table 1. First through third instars molted at comparable intervals after feeding (ca. 11 days). Fourth instars required slightly longer periods of time to molt than the younger instars (ca. 13 days). Fifth instars required the longest period of time before ecdysis occurred (ca. 20 days).

Bugs could be fed experimental blood meals containing other miscellaneous components using an alternative system. Water-jacketed glass feeders were supported by a special Plexiglas structure and interconnected with tubing; warm water was pumped through the system of feeders from a warmed water bath (36-40° C) (Fig. 2). As the glass feeders were bell-shaped, the opening bottom was covered by a stretched membrane. This created an enclosed space that contained the blood meal, which was introduced into by a long glass pipette by the long neck of the feeder. Blood meals were prepared beforehand and administered just before feeding occurred. Up to eight feeders could be operated at the same time.

Blood meals were constantly stirred at approximately 100 rpm. A small propeller, consisting of a wooden applicator stick and masking tape, was inserted into each feeder. The opposite end of the applicator stick was firmly inserted into a rubber stopper, which contained a small magnet. The top of the structure supported a removable magnetic stirring plate with twelve individual magnetized surfaces. Hence, each of the eight feeders could be stirred at a uniform and controlled speed. Previous models used variable speed drills suspended above the glass feeders or utilized a network of small motors attached to propellers. The former was too costly, loud and required a great deal of space; the latter could not easily achieve uniformity of stirring for each feeder.

After all blood meals had been added and were being stirred, prepared populations of bugs were introduced beneath each feeder and permitted to feed to repletion. Each set of bugs was placed in a jar or cup containing plentiful amounts of filter paper, which allows the bugs to climb to the surface to feed and to absorb the vast amount of urine and feces following a heavy blood meal.

Table 1. Mean amount of time to ecdysis required by *Rhodnius prolixus* after one full blood meal.

Life Stage	Mean Elapsed Time to Ecdysis	Range (days)
Eggs	13.84 ± 0.03	13-15
1st instars	11.31 ± 0.20	9-17
2nd instars	11.31 ± 0.25	9-22
3rd instars	11.23 ± 0.17	9-22
4th instars	13.49 ± 0.20	12-19
5th instars (male)	20.46 ± 0.32	19-25
5th instars (female)	20.33 ± 0.30	18-23

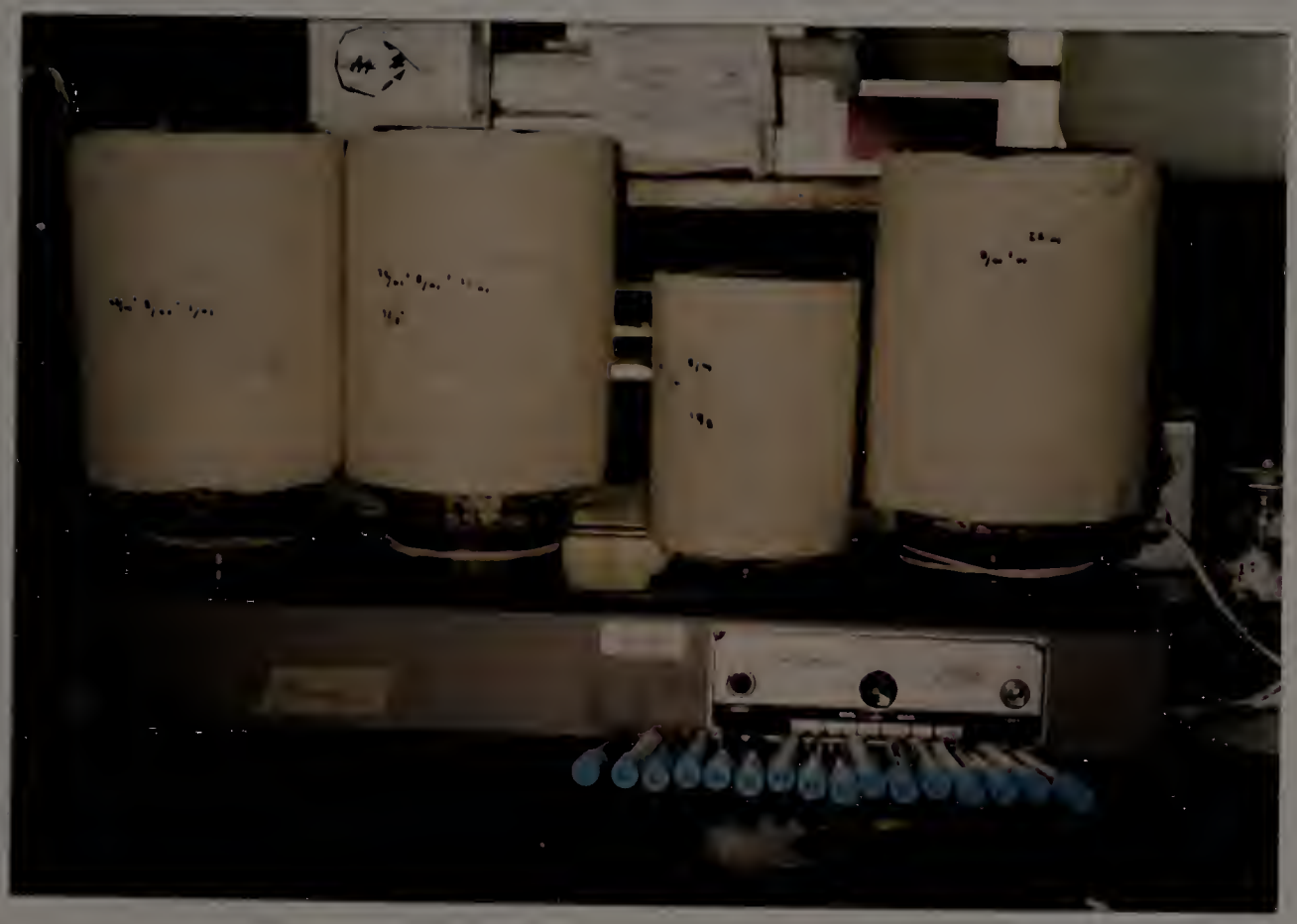

Figure 1. Colony maintenance of *Rhodnius prolixus* using heat-sealed parafilm envelopes, filled with citrated rabbit blood, placed on a slide warmer.

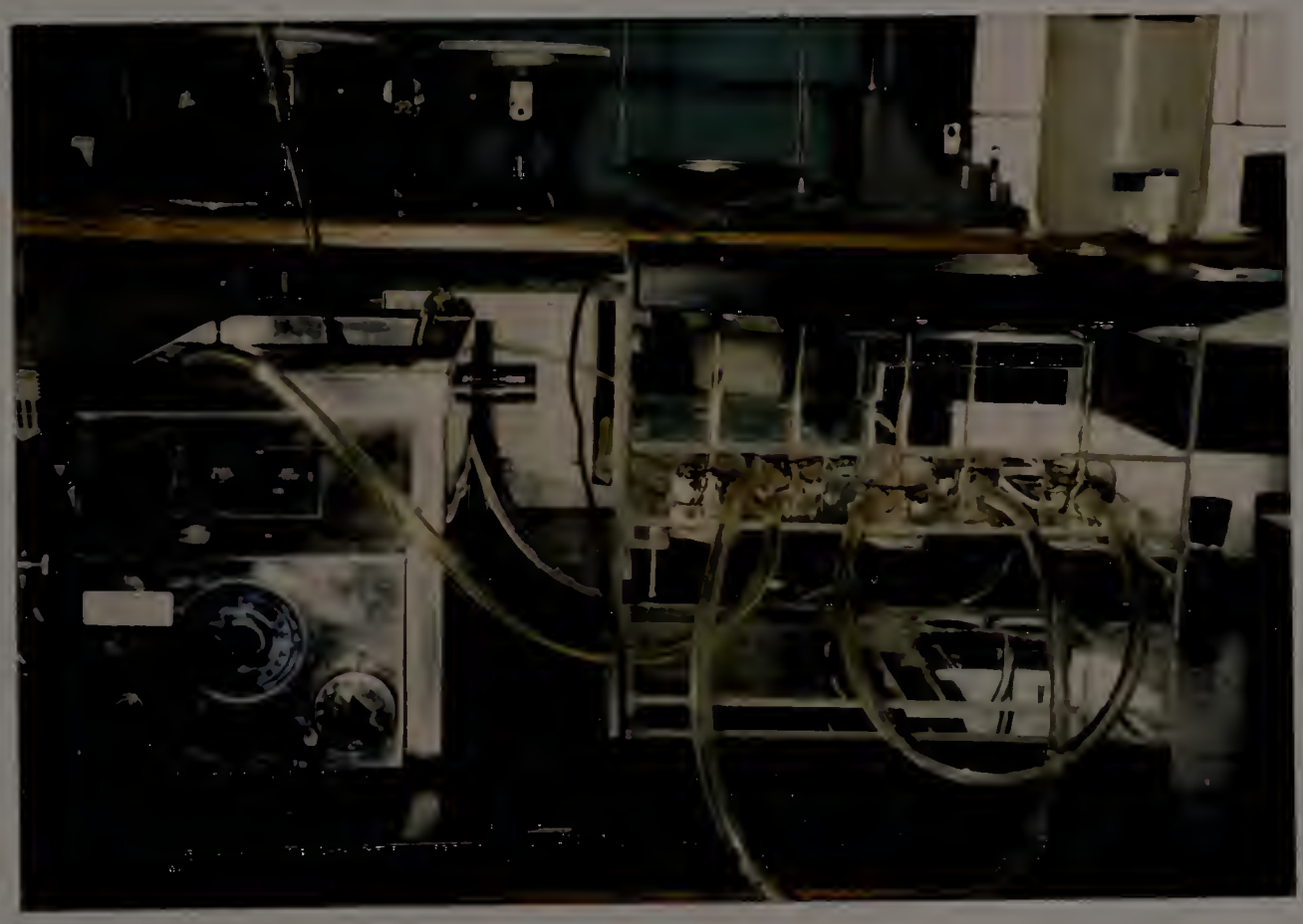

Figure 2. Artificial membrane feeding system used for *Trypanosoma cruzi* infection of *Rhodnius prolixus*.

CHAPTER III

TRANS-SIALIDASE INTERACTION WITH *TRYPANOSOMA CRUZI* IN *RHODNIUS PROLIXUS*

Introduction

The exact factors involved in *T. cruzi* invasion of mammalian cells are unknown and it is more likely that invasion is attributable to a combination of factors, rather than one factor alone. Three parasite-produced proteins suspected to be involved in host-parasite interactions are penetrin, fibronectin, and *trans*-sialidase (TS). Penetrin has been implicated to have a role in trypomastigote attachment to mammalian cells while antibodies to fibronectin successfully prevent trypomastigote invasion (Burleigh and Andrews, 1995). Chuenkova and Pereira (1995) discovered that exogenous *Trypanosoma* TS and recombinant TS enhanced *T. cruzi* virulence in mice. Pereira (1996) later provided evidence that only 20-30% of Silvio parasites produce TS (TS^+ parasites). However, TS^+ parasites can transform into TS^- parasites. TS^+ parasites were more highly invasive than TS^- parasites in mice.

Although pathogenic in mammals, infection by *T. cruzi* is not considered to have serious deleterious effects on its kissing bug host (Schaub, 1988, 1992). However, Lima et al. (1992) reported that reproduction of *T. cruzi*-infected *Panstrongylus megistus* was significantly reduced when compared to uninfected bugs. It has been observed in the laboratory that *T. cruzi*-infected *R. prolixus* required slightly longer periods of time to develop before ecdysis occurs.

Impact of TS alone on invertebrate host is unknown. Amino et al. (1995) discovered that in the gut homogenates of *Triatoma infestans*, *T. cruzi* parasites were poorly sialylated. This fact was attributed to an innate sialidase activity existing in the bug gut and caused the researchers to surmise that *T. cruzi* did not require sialic acid in order to survive or develop in the kissing bug vector. Thus, the role of TS in *R. prolixus* was studied to answer the following questions:

(1) Does TS affect the life history parameters of *R. prolixus*?

(2) Do TS types affect the development of *T. cruzi* parasite loads in *R. prolixus?*

(3) What is the role of TS phenotypic expression on *T. cruzi* parasite load in *R. prolixus?*

(4) Does the period of fasting significantly affect *T. cruzi* development in *R. prolixus*?

Materials and Methods

Insects

Rhodnius prolixus were maintained in the laboratory at 28° C, 80% humidity, and a 14L:10D photoregime. Insects were separated by instar in large glass jars and fed citrated rabbit blood approximately every other week. Experimental insects were selected approximately 2-3 weeks after a blood meal. Insects that took a blood meal and molted to the next stage were easily identified.

Fourth instars were starved for 3-5 weeks after molting before they were fed an infective blood meal containing *T. cruzi*. Parasite concentrations were adjusted so that the average insect would ingest approximately 7500 parasites within one replete blood meal. Insects failing to feed or to molt into 5th instars were discarded from the study.

Blood

Citrated (129 mM) rabbit blood was used as the blood meal in all experiments. Rabbit blood was purchased from a local vendor (Millbrook Farms), who collected rabbit blood from an ear vein and delivered it in a vacutainer the next day to the Medical Entomology Laboratory at the University of Massachusetts. The blood to be infected with *T. cruzi* was pooled into a larger tube and transported, on ice, to Tufts University.

Parasites

Trypanosoma cruzi trypomastigotes (Silvio strain) were obtained from Miercio Pereira of Tufts University. Parasites at Tufts University are maintained at 37° C and passed through Vero cell cultures daily. Cultures containing trypomastigotes are washed once or twice with 10% fetal calf serum-Roswell Park Memorial Institute (RPMI) media and centrifuged at 1000 gravities x 10 min. Trypomastigotes from the culture were counted and an appropriate volume containing the correct number of desired parasites was added to the blood. Infected blood was packed on ice and transported back to the Medical Entomology Laboratory for immediate use in an experiment.

Trans-sialidase

TS types were provided by Tufts University. *Trypanosoma* TS was purified from trypomastigote cultures while the recombinant TS types (19y rTS and rTS2000) were produced by transformed *Escherichia coli* XL-1 containing TS inserts. Purification of recombinant forms of TS was done in a manner identical to that of *Trypanosoma* TS: the enzyme was bound to a monoclonal antibody as the Silvio strain parasites were passed

through the affinity column, eluted with the synthetic peptide TR and then separated from the TR peptide by fast protein liquid chromatography (Chuenkova and Pereira, 1995).

TS types were maintained on ice until incorporated into individual blood meals. Once the enzyme had been added to the blood meal, it was administered as quickly as possible to the insects.

Trans-sialidase specific activity assay

Trans-sialidase activity was determined in the following reaction mixture combined in the respective order in a 1.5 ml microcentrifuge tube: 12 µl distilled water, 6 µl bovine serum albumin (BSA) (10 mg/ml), 12 µl 50 mM Tris-HCl (pH = 7.2), 10 µl fetal calf serum (FCS), 20 µl bug gut extract, and 10 µl ^{14}C-lactose (40-50,000 cpm). Control tubes contained the same materials in equal volumes with the single exception that 20 µl of distilled water was added instead of 20 µl of bug extract (obtained by centrifugation of bugs). Once all of the materials had been added, the mixture was vortexed briefly and incubated at room temperature for approximately 21 hr. The reaction was stopped by the addition of 1 ml of water per reaction tube.

Each reaction mixture was transferred to an ion exchange reaction column containing a glass bead; the tip was loaded with the ion exchange resin (2 g QAE Sephadex A-50 in 200 ml distilled water) until a 1 ml column bed volume was formed.

The unbound components following the TS reaction were eluted with five aliquots of 1 ml distilled water. Bound TS components were eluted with 1 ml 1 M NaCl. Eluate was transferred to a scintillation vial containing 2 ml EcoScint scintillation fluid and

shaken briefly. Vials were placed in a β-liquid scintillation counter to determine radioactivity.

Confirmation of *Trypanosoma trans*-sialidase entry into the bug vector

Groups of bugs were permitted to feed for 30 min on blood meals containing a range of TS activity (2,337, 14,694, 29,388, and 58,444 total cpm enzyme activity/ml blood). Insects were then placed in an incubator set at 28° C and 80% humidity.

Within 12-15 hr post-ingestion, 4 live bugs from each group were removed and anesthetized with carbon dioxide for post-ingestion TS assays. Unfed bugs were used as an additional control. Each bug had the posterior section of their abdomen removed with a pair of microscissors and centrifuged at 492 gravities for 1 min to collect the gut contents. The volume collected was approximately 25 µl per bug and contained a small but inevitable amount of hemolymph. Gut extracts were incubated in the TS reaction mixture overnight and assayed for radiometrically the following morning.

Duration of 19y rTS *trans*-sialidase activity

Activity of recombinant 19y TS, genetically identical to *Trypanosoma* TS except it is produced by a recombinant bacterium, was assayed as detailed previously for several days after ingestion by starved, uninfected 4th instars, until enzyme activity was no longer detected.

Fourth instars, starved 3-5 weeks after molting, were fed on blood meal diets of various composition, administered through membrane-covered, water-jacketed glass feeders. Blood meals varied in TS activity and consisted of the following concentrations:

244, 1466, 2931, and 5836 total cpm enzyme activity/ml blood. Blood fed to control groups did not contain TS.

Approximately every 24 hr after the initial feeding, 2 bugs were removed from each group and assayed for post-ingestion TS activity. After 6 days, assessment of enzyme activity was reduced to every other day until activity was comparable to background levels. The tip of the bug abdomens were snipped with a pair of microscissors and placed in individual 1.5 ml microcentrifuge tubes containing false aluminum mesh bottoms and 20 µl 10% FCS-RPMI containing antibiotics. Tubes were centrifuged at 492 gravities for 1 min and crude gut extracts were collected at the bottom. Gut extracts were incubated in the TS activity reaction mixture for 21 hours and TS activity determined radiometrically as before. Samples were taken and measured as long as enzyme activity was detectable (above background cpm values of control).

Trans-sialidase impact on adult female fecundity and mortality

This study was limited to mated female *R. prolixus*, since previous research had demonstrated that mated females laid significantly more eggs than virgin females (Chiang and Chiang, 1995). Fifth instar *R. prolixus* were separated by sex by examining the posterior portion of the abdomen (females have a posterior cleft and the tergites were closer together than in males) and maintained in separate jars for males and females, to assure virgin status at emergence. Males were fed three times over the course of several weeks before being paired with unfed virgin females in separate vials. After one week of pairing, males were removed from the vial before females were fed for the first time as adults. Approximately half (n = 21) of the females were permitted to feed on a blood meal

containing *T. cruzi* TS (57,142 total cpm/ml blood) and the remaining half (n = 20) were fed a blood meal with only phosphate buffered solution (PBS) added.

Before feeding, each female was weighed and identified with a label, secured around the female's thoracic/abdominal area. Females were weighed again, after the label was attached. All labeled females were placed together in a jar designated for a particular blood meal (with or without TS) and permitted to feed to repletion. Fed individuals were separated, weighed and returned to their vials.

Females were monitored for egg laying activity for 26 days. On day 26, all females were dissected to look for developing eggs and the total number of eggs produced by the single blood meal was determined. An E-value was calculated as follows (Chiang and Davey, 1990):

$$\text{E-value} = \frac{\text{Total no. eggs laid by day 26}}{(\text{initial weight} \times \text{blood meal weight}) \times 1000}$$

Female mortality and fecundity differences between treatments were statistically analyzed with a Student's t-test ($p < 0.05$).

Trans-sialidase impact on nymphal molting and mortality

Starved fourth instars were fed various concentrations of TS (ranging from 14,667 to 955,700 cpm/ml blood) and monitored for 33-47 days post-ingestion for molting activity and mortality. Data were analyzed by a one-way ANOVA and considered significant at levels of $p < 0.05$. Experiments were then compared with one another to look for consistent effects.

Impact of *Trypanosoma trans*-sialidase on *Trypanosoma cruzi* parasite load in *Rhodnius prolixus*

A series of five experiments were performed, in which starved 4th instars ingested various concentrations of *Trypanosoma* TS within an infective blood meal. The enzyme concentrations tested ranged from 2,337 to 955,700 cpm/ml. Insects were observed for 33-47 days before they were dissected and parasite counts recorded.

In this and all subsequent experiments, a standardized procedure for blood meal preparation and feeding was followed. Each blood meal contained enough parasites for an individual bug to ingest approximately 7500 trypomastigotes. Insects were allowed to feed on their blood meals for 30 min. Bugs failing to feed to repletion were discarded. Remaining insects were observed daily for 33 days to record mortality and molting activity. On day 33, insects were chilled and dissected live in PBS: four hindgut/rectum samples were removed and homogenized together in a 1.7 ml microcentrifuge tube. After live "wiggler" counts (total number of metacyclic trypomastigotes and epimastigotes) were determined using a hemacytometer (10 µl of sample material), parasite samples were further processed and purified for stained slide preparation (Diff-Quik®) to determine differential parasite counts.

Discrimination between metacyclic trypomastigote (metacycs) and epimastigote (metacycs) morphological forms was based on the location of the kinetoplast in relation to the nucleus. Parasite loads were quantitated by metacyclic trypomastigote (T count) and epimastigote (E count) infection levels and based on one hemacytometer count (10 µl of

sample). Data were analyzed with one-way ANOVAs and considered to be significant only below levels of $p < 0.05$.

$$\text{T count} = \text{wigglers} \times \frac{\text{metacycs}}{\text{metacycs} + \text{epis}} \qquad \text{E count} = \text{wigglers} \times \frac{\text{epimastigotes}}{\text{metacycs} + \text{epis}}$$

Impact of 19y rTS *trans*-sialidase on *Trypanosoma cruzi* parasite load

Recombinant 19y rTS is genetically identically to *Trypanosoma* TS, but produced by a bacterium. Ten percent of the total recombinant TS activity was used, in comparison with previous experiments using *Trypanosoma* TS, since the former tends to have a 10-fold higher protein content than the latter. Over a series of three experiments, starved fourth instars were fed various concentrations of 19y rTS (ranging from 550 to 5828 cpm/ml blood) with *T. cruzi*-infected blood meals and observed for 33 days. On day 33, parasite counts were recorded and stained slides were analyzed for the proportion of metacyclics and epimastigotes as described earlier. Insects ingesting 550 cpm/ml blood were fasted for three different periods of time to examine whether fasting period affects TS and *T. cruzi* interaction within the insect.

Impact of rTS2000 *trans*-sialidase on *Trypanosoma cruzi* parasite load

Recombinant rTS2000 TS, which lacks the series of carboxyl-terminus tandem repeats of amino acids, that are critical for enzyme oligomerization. In two experiments, bugs ingested various levels of rTS2000 TS (ranging from 1469 to 5834 cpm/ml blood)

simultaneously with an infective blood meal. After 33 days of observation, bugs were dissected and parasite counts recorded.

Impact of *trans*-sialidase antibodies on *Trypanosoma cruzi* parasite load

The IgG1 murine monoclonal antibody (mAb) TCN-2 is specific for the amino acid tandem repeats located at the carboxyl-terminus of TS. In the mammalian system, the addition of TCN-2 to *T. cruzi* parasites increases parasitemia. Since the impact of TCN-2 on *T. cruzi* infection in the insect vector was unknown, various concentrations of TCN-2 were introduced into the insect. Experimental blood meals were administered through four successive experiments at the following concentrations: 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, and 50.0 μg mAb/ml blood. Control blood meals contained an IgG1 murine monoclonal antibody specific for spectrin (Maine Biotechnologies).

To confirm that experimental results were due to the action of antibody binding to the carboxyl-terminus, polyclonal TS antibody derived from the Montalvania-13 (MV-13) strain was added to infective blood meals at the concentration 1.0 μg/ml blood. This antibody binds to the same site as that of TCN-2, but is produced by another *T. cruzi* strain and has less binding specificity.

Effects of fasting period on parasite load

Due to variability of previous experimental results, I examined whether the fasting period of nymphs exerts any impact *T. cruzi* development. Three separate experiments were conducted in which 4th instars were starved (after molting) for varying periods

before infected with *T. cruzi*: (1) 1, 4, and 8 wk, (2) 1, 8, and 16 wk, or (3) 3 and 5 wk. Bugs were dissected 33 days post-blood meal, to determine parasite load.

Results

Confirmation of *Trypanosoma trans*-sialidase entry into the bug vector

Every experimental dilution (TS diluted by PBS up to dilutions as high as 1:1000) that was tested for TS activity and allowed to incubate overnight produced positive results.

All bugs that ingested a blood meal containing TS also ingested the active enzyme (Fig. 3). Though variation of means among the groups were statistically significant (p-value = 0.0002), sample sizes were insufficient to detect statistical significance between groups. However, my goal to confirm the presence of post-ingestion TS activity in the bug gut was achieved.

Interestingly, bugs that ingested TS within an uninfected blood meal had the highest amount of TS activity (though not significantly higher). This is surprising, because overall, bugs ingesting only TS were higher than those for bugs ingesting *T. cruzi* with or without TS. Abdominal extracts of bugs ingesting parasites with or without TS should have produced values higher than those of any uninfected bug, due to the natural additional production of *trans*-sialidase by the recently-ingested trypomastigotes themselves.

Duration of 19y rTS *trans*-sialidase activity

The lowest levels of rTS activity could not be detected on any day, not even in the purified 19y rTS standards (Fig. 4). All other rTS levels remained detectable until day 10, but were comparable to background levels by day 12 (Fig. 4). Apparently, 19y rTS remains catalytically active in the bug for at least 10-12 days.

Trans-sialidase impact on adult female fecundity and mortality

There were no significant fecundity differences between E-values of females ingesting TS or PBS (Fig. 5). Though females ingesting TS did have slightly lower E-values, this difference was not significant. There also were no significant differences in adult mortality.

Trans-sialidase impact on nymphal molting and mortality

There was no consistent effect on molting activity or mortality of nymphs between experiments, although infected bugs required slightly longer periods to molt than uninfected bugs. This difference, however, was not significant ($p > 0.05$).

Impact of *Trypanosoma trans*-sialidase on *Trypanosoma cruzi* parasite load

The addition of exogenous *Trypanosoma* TS failed to have a consistent impact on *T. cruzi* parasite load within the insect vector (Table 2).

Impact of 19y rTS *trans*-sialidase on *Trypanosoma cruzi* parasite load

Recombinant 19y rTS did not consistently affect *T. cruzi* parasite load in the insect vector (Table 3).

Impact of rTS2000 *trans*-sialidase on *Trypanosoma cruzi* parasite load

Recombinant rTS2000 consistently increased the parasite load of *T. cruzi* within *R. prolixus* in two experiments (Table 4).

Impact of *trans*-sialidase antibodies on *Trypanosoma cruzi* parasite load

All blood meal concentrations of TCN-2 greater than 0.005 μg/ml blood produced significantly higher parasite loads than that produced by the IgG1 control blood meal (Figs. 6-8). I surmised that the optimal concentration of TCN-2, consistently producing the highest *T. cruzi* parasite load, was 1.0 μg/ml blood. Confirmatory studies with TS polyclonal antibodies demonstrated that the additional of this antibody significantly increased parasite loads in *R. prolixus* (Fig. 9).

Effects of fasting period on parasite load

Results were inconsistent between the three experiments (Figs. 10-12), suggesting that fasting period is not the sole factor in determining *T. cruzi* parasite load in *R. prolixus*. More importantly, *Trypanosoma cruzi* appears to be able to thrive in insects fasted for extended periods of time. This suggests that *T. cruzi* has the ability to survive in an environment that has been depleted of sialic acids (donated by a fresh blood meal).

Table 2. Impact of *Trypanosoma trans*-sialidase (TS) on *Trypanosoma cruzi* parasite load in 5th instars after 33 days post-blood meal.

TS Concentration (total cpm/ml blood)	Trial #1[a]	Trial #2[a]	Trial #3[b]	Trial #4[b]	Trial #5[b]
		Total No. Wigglers/Bug/Hemacytometer Count ± SEM			
2,337		1.1 ± 0.4			
7,500					100.6 ± 8.5
14,694		2.5 ± 1.1	68.3 ± 4.5		
18,000				31.5 ± 31.5	
29,300		11.0 ± 3.5	13.9 + 11.6		
37,500					98.4 ± 5.5
58,500		5.3 + 2.4	0.4 ± 0.1		
238,925	1.0 ± 1.0				
477,850	0.0 ± 0.0				
955,700	0.0 ± 0.0				
Control	2.7 ± 2.7	1.6 ± 0.2	61.0 ± 2.9	26.3 ± 14.3	28.8 ± 2.3

[a] = monitored for 47 days before dissection
[b] = monitored for 33 days before dissection

Table 3. Impact of 19y rTS *trans*-sialidase (TS) on *Trypanosoma cruzi* parasite load in 5th instars after 33 days post-blood meal.

TS Concentration (total cpm/ml blood)	Trial #1	Trial #2	Trial #3	Trial #4	Trial #5
		Total No. Wigglers/Bug/Hemacytometer Count ± SEM			
550 (1 wk starved)		40.6 ± 3.5			
550 (4 wk starved)			47.8 ± 7.9		
550 (8 wk starved)				59.2 ± 6.5	
1025					67.5 ± 5.6
1471	3.8 ± 2.3				
2942	18.1 ± 1.6				
5828	8.6 ± 3.9				
Control	3.5 ± 1.4	35.1 ± 4.0	53.5 ± 2.7	33.4 ± 3.0	28.8 ± 2.3

Table 4. Impact of rTS2000 *trans*-sialidase (TS) on *Trypanosoma cruzi* parasite load in 5th instars after 33 days post-blood meal.

TS Concentration (total cpm/ml blood)	Trial #1	Trial #2
	Total No. Wigglers/Bug/Hemacytometer Count ± SEM	
1469	20.6 ± 4.3	
1623		56.9 ± 3.6
2938	18.6 ± 1.4	
5834	18.9 ± 2.1	
Control	4.5 ± 1.5	28.8 ± 2.3

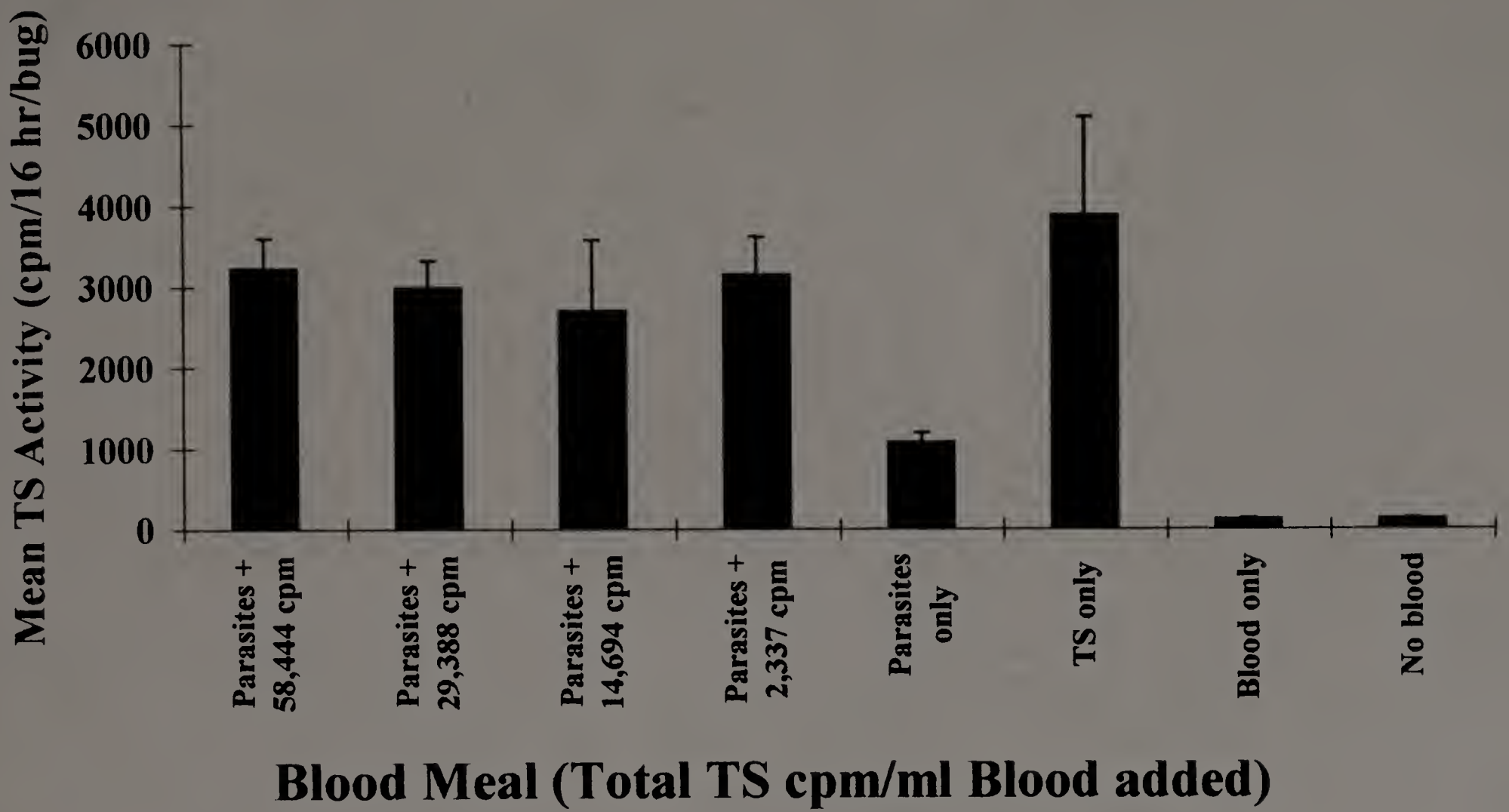

Figure 3. Mean TS activity (± SEM) detected in 4th instar *Rhodnius prolixus*.

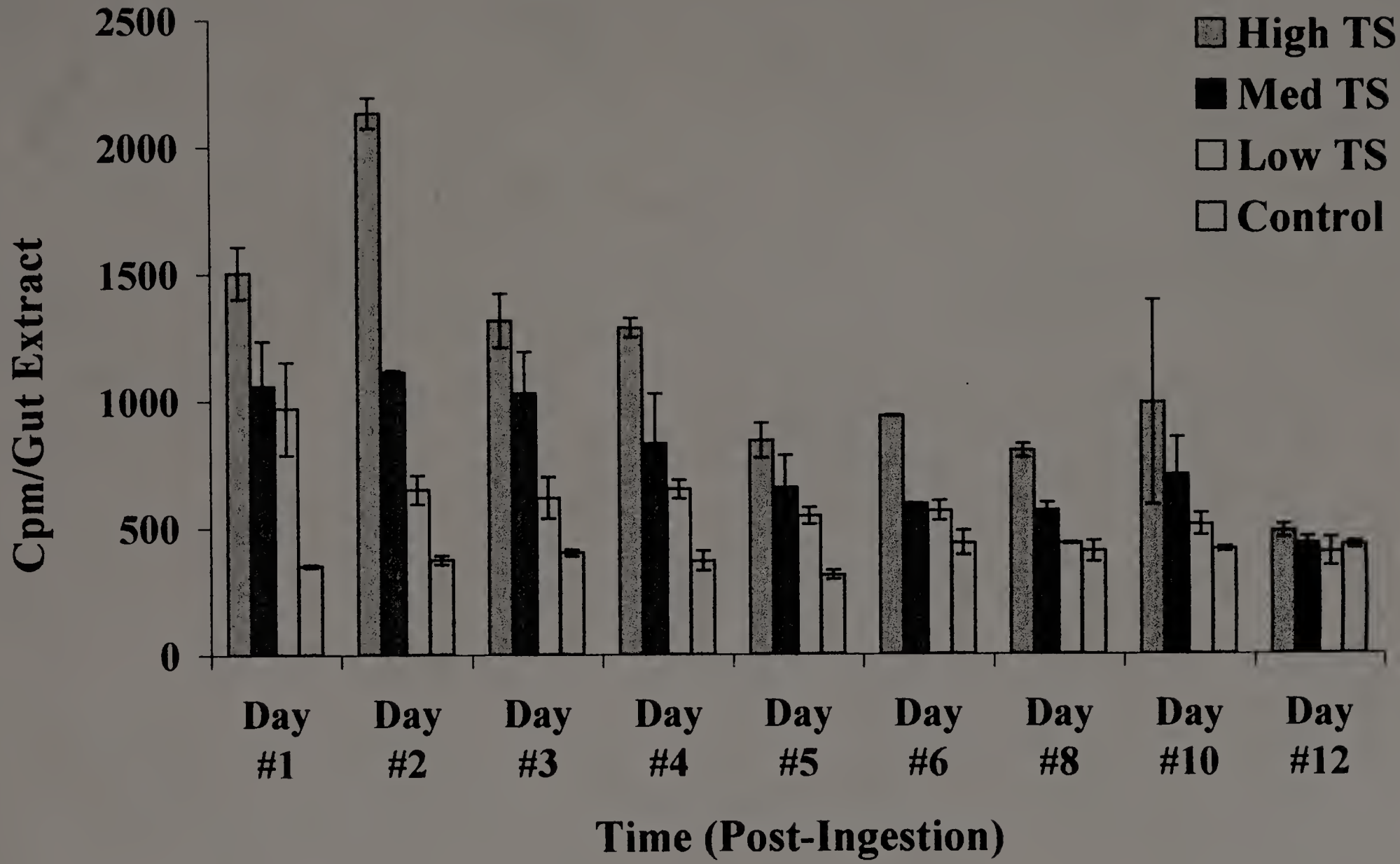

Figure 4. Mean TS activity (counts per minute ± SEM) detected per bug gut extract.

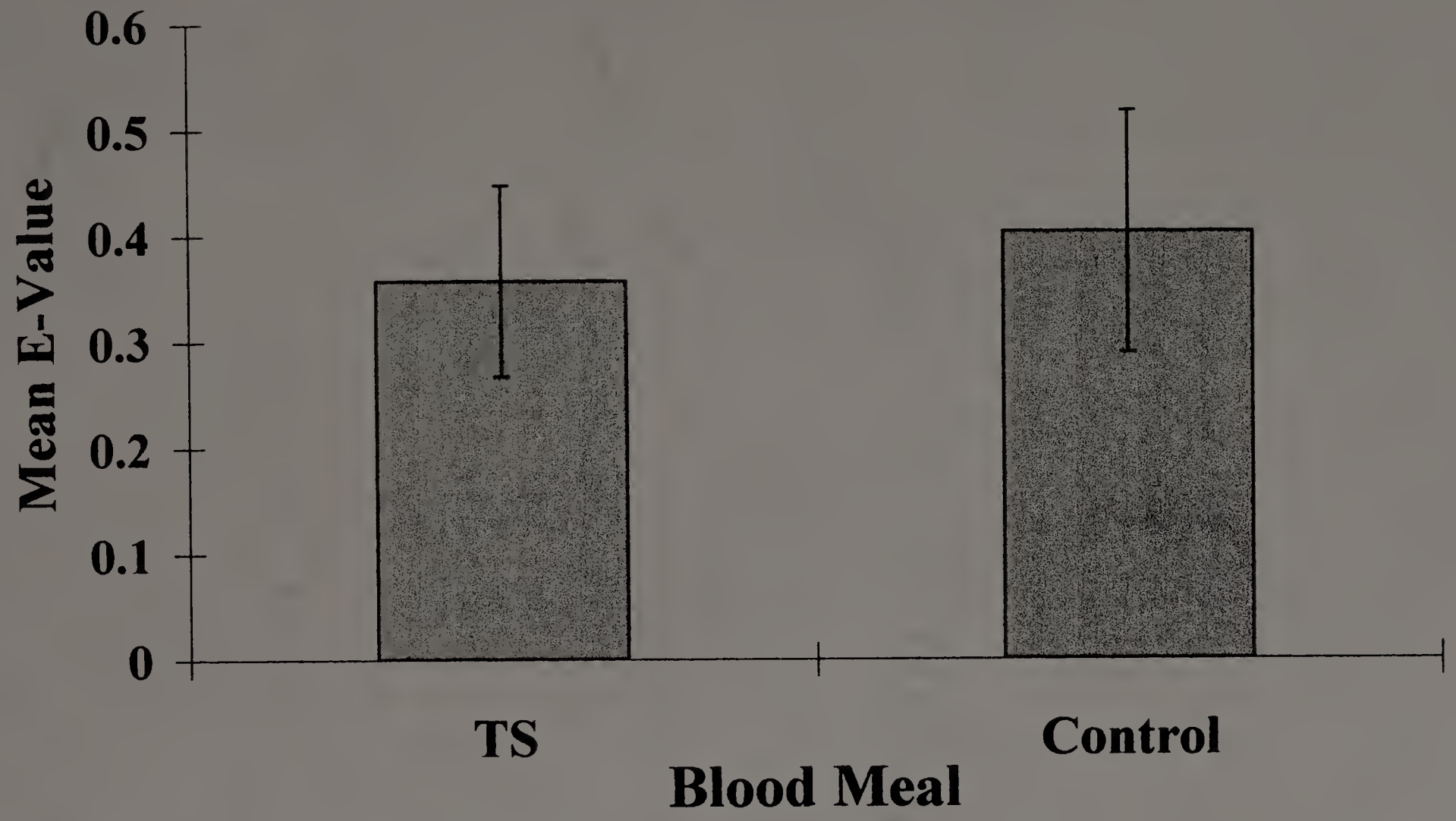

Figure 5. Mean E-values (± SEM) for mated females ingesting TS (57,142 total cpm/ml blood). E-value = (total number of eggs laid by day 26/(initial weight x blood meal weight) x 1000)

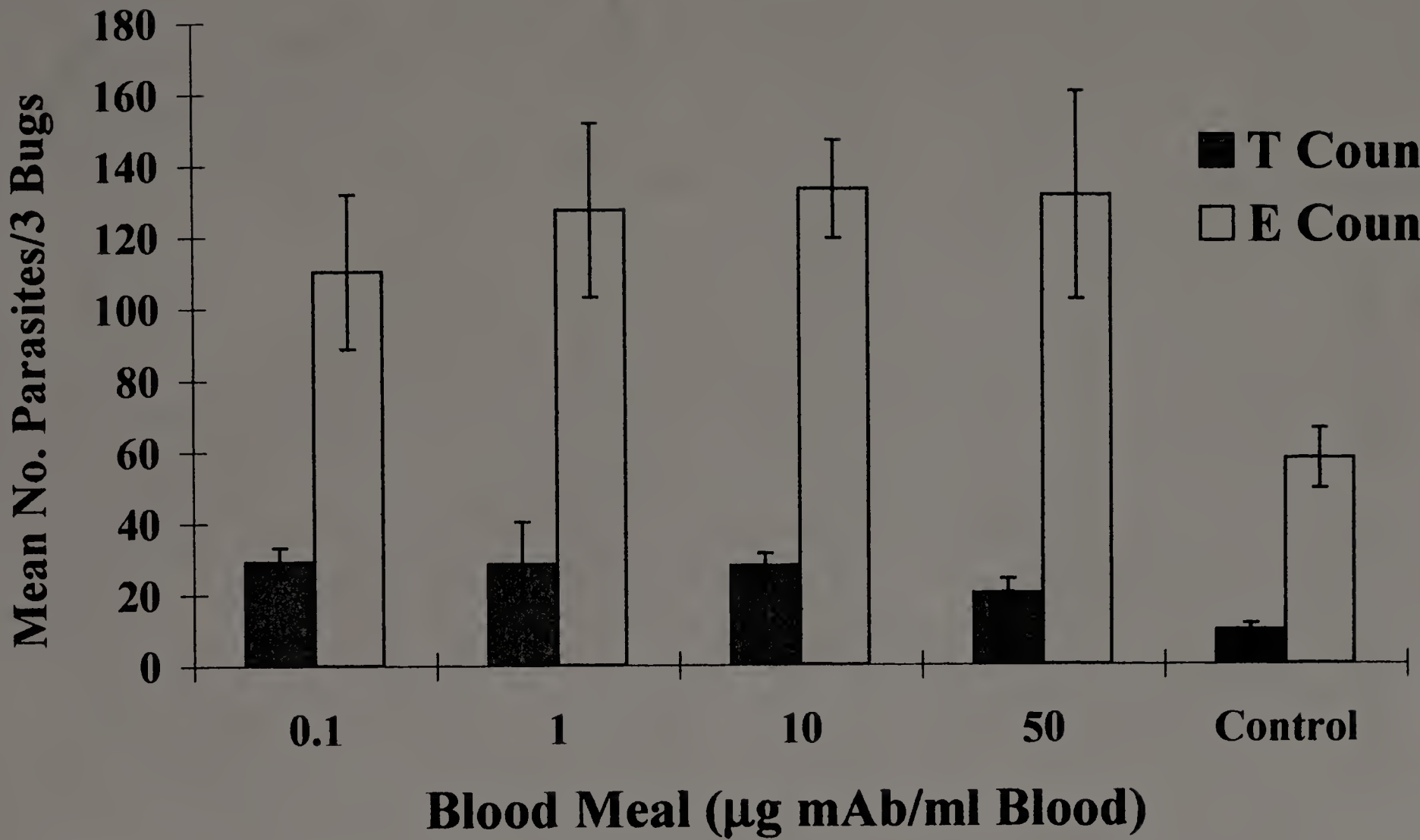

Figure 6. Impact of TCN-2 concentration (0.1-50 µg mAb/ml blood) on *Trypanosoma cruzi* parasite load (± SEM) (33 days post-ingestion). (T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

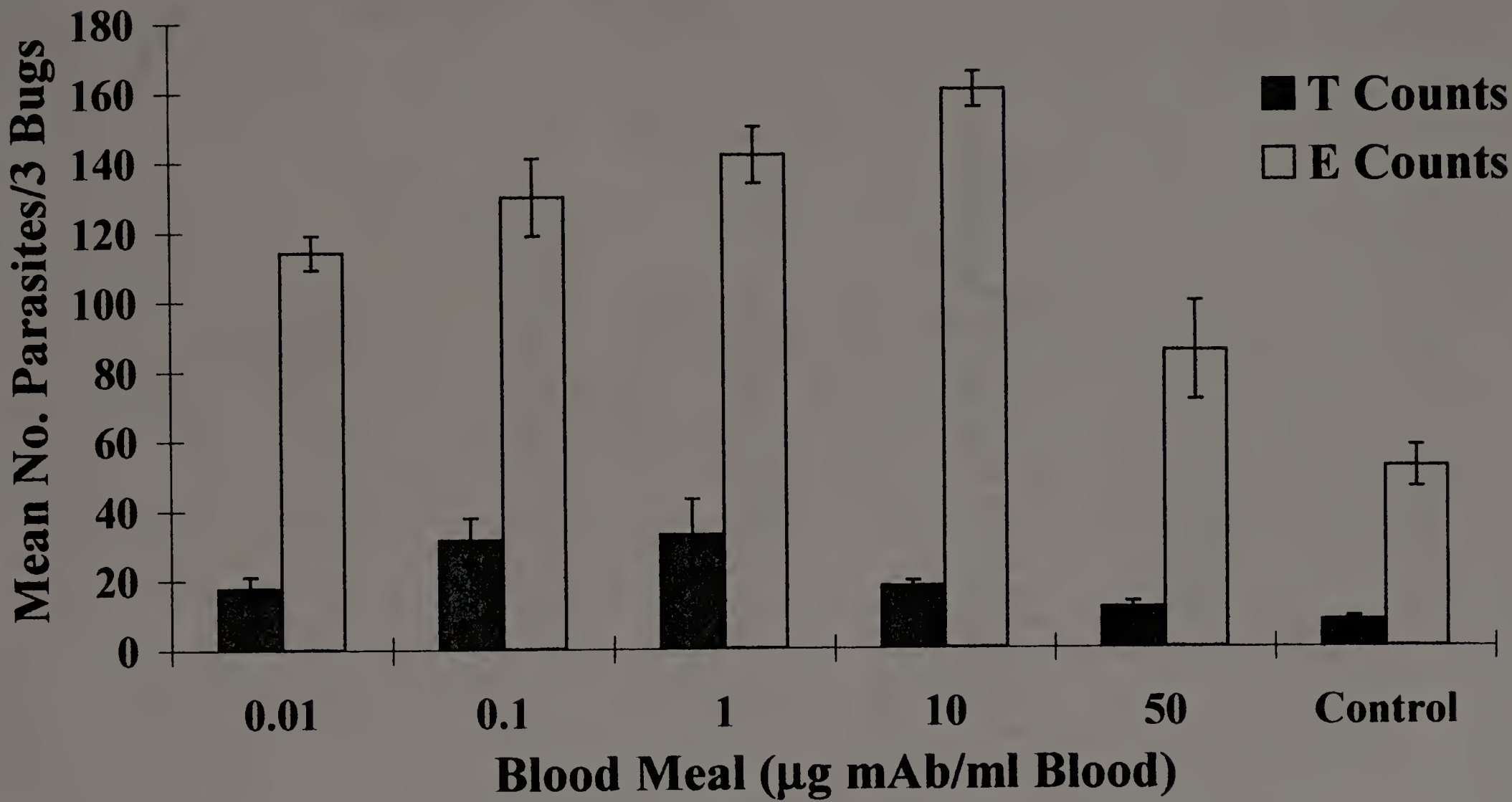

Figure 7. Impact of TCN-2 concentration (0.01-50 µg mAb/ml blood) on *Trypanosoma cruzi* parasite load (± SEM) (33 days post-ingestion). (T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

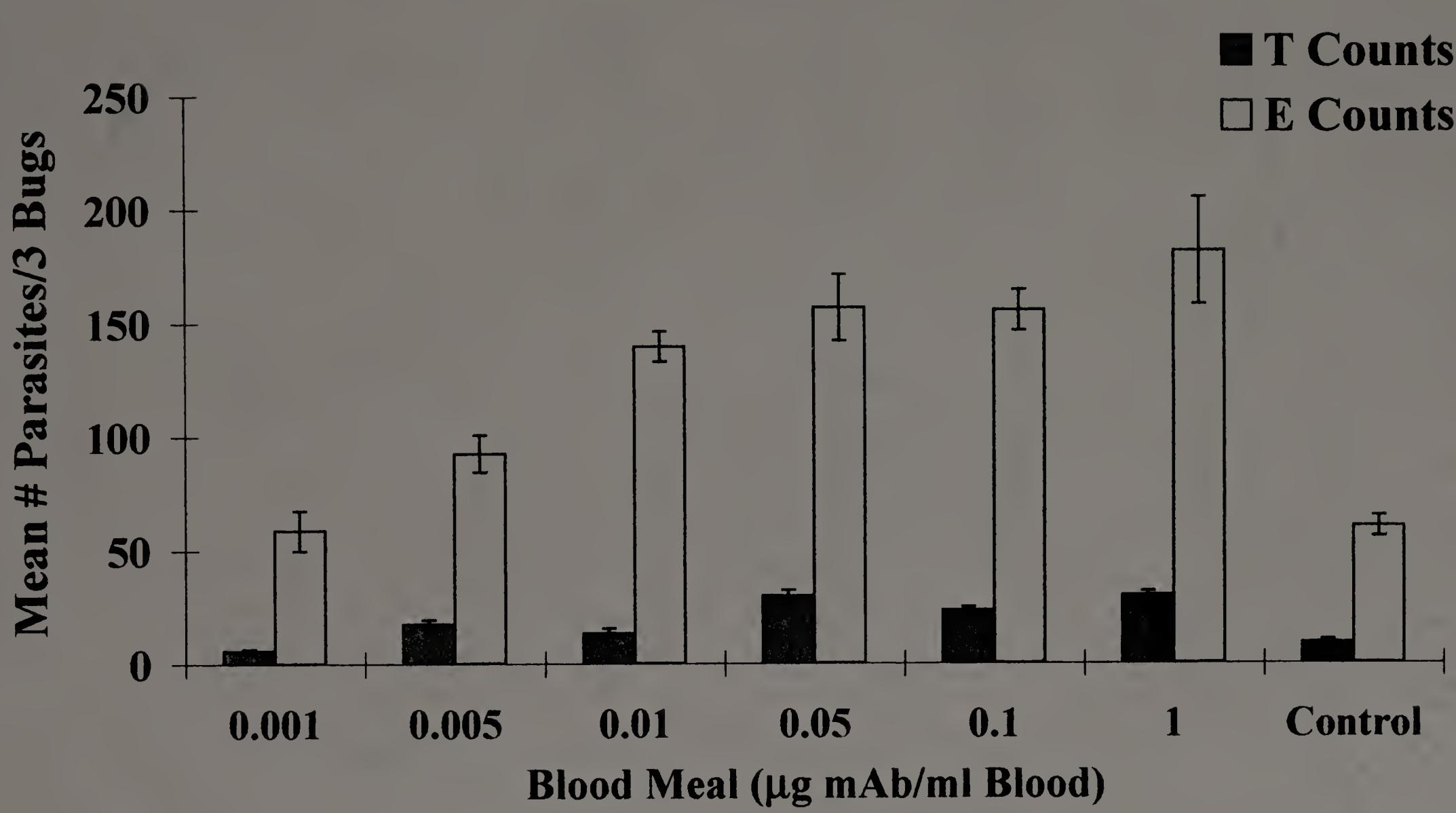

Figure 8. Impact of TCN-2 concentration (0.001-1.0 µg mAb/ml blood) on *Trypanosoma cruzi* parasite load (± SEM) (33 days post-ingestion). (T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

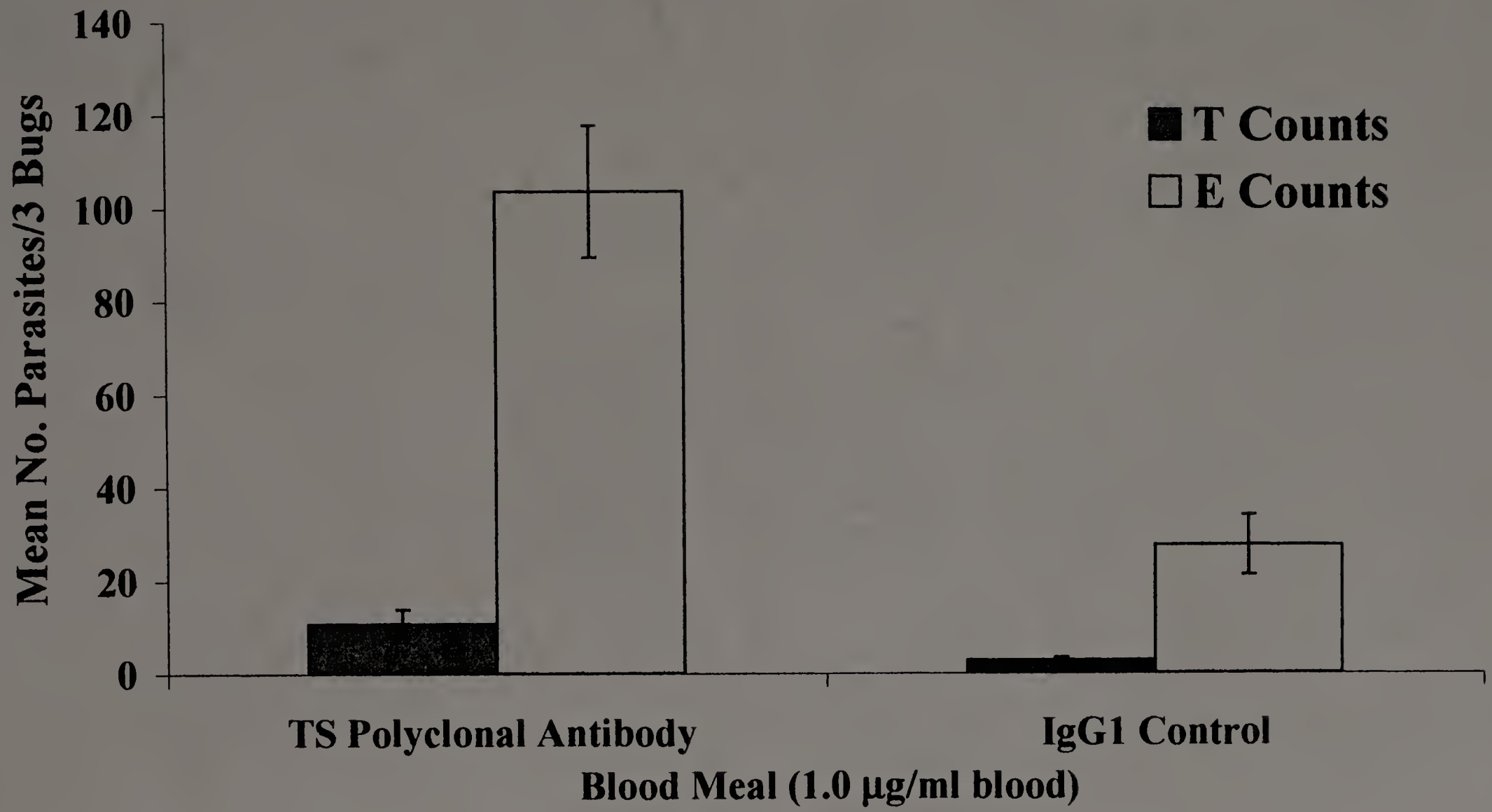

Figure 9. Impact of TS polyclonal antibody (1.0 µg Ab/ml blood) on *Trypanosoma cruzi* parasite load (± SEM) (33 days post-ingestion). (T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

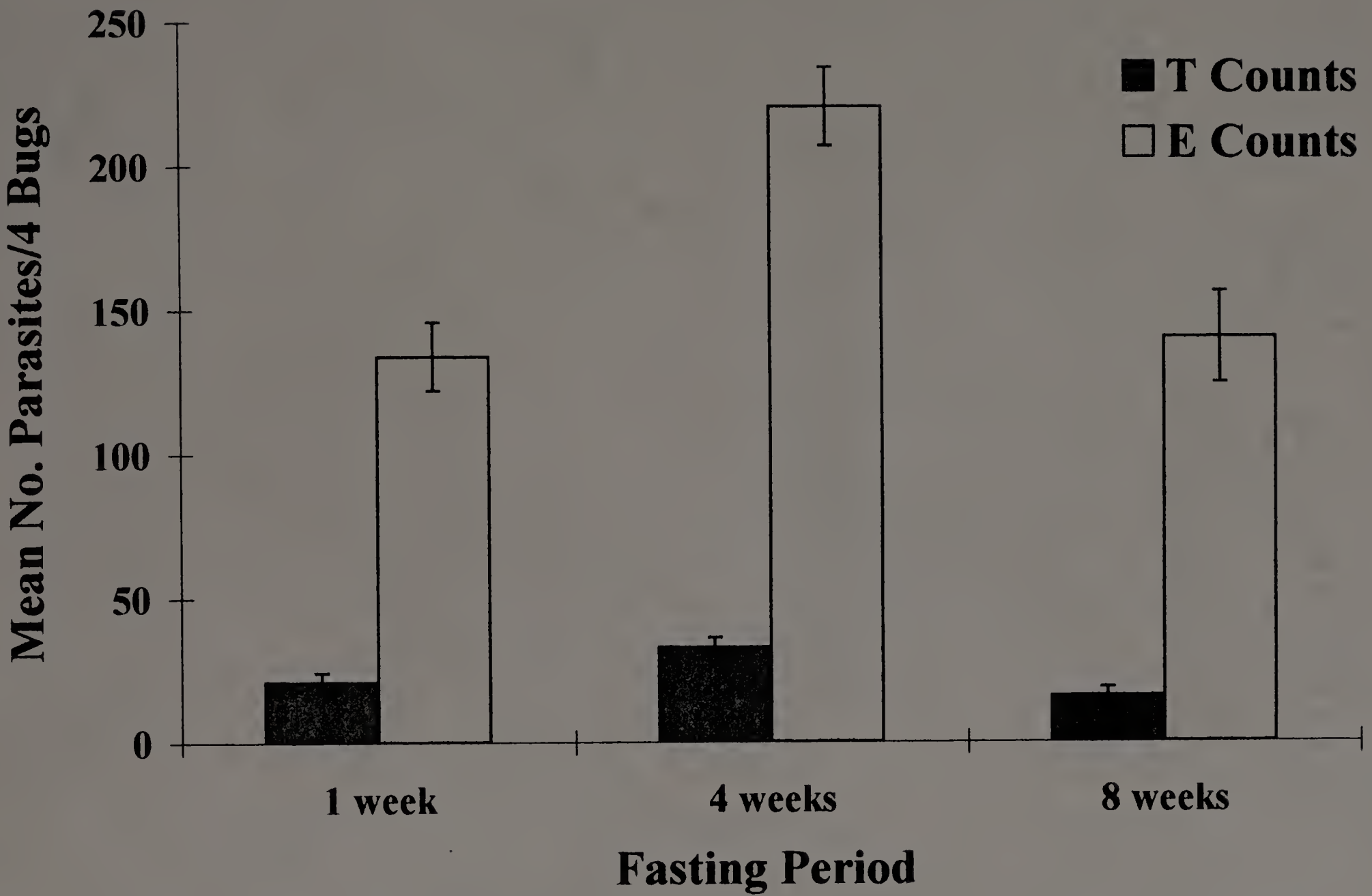

Figure 10. Impact of fasting period (1, 4, or 8 wk) on *Trypanosoma cruzi* parasite load (± SEM) (33 days post-ingestion). (T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

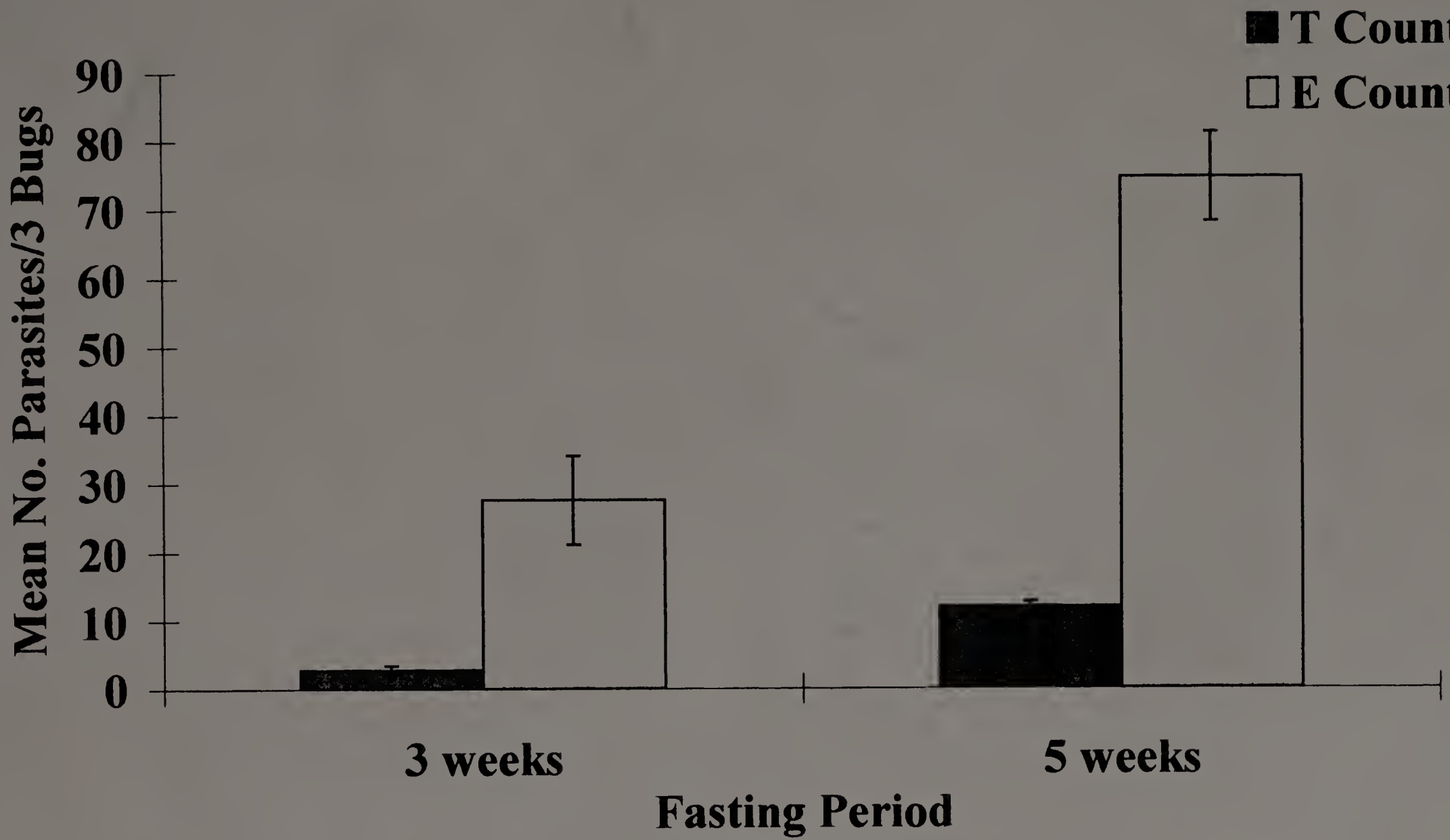

Figure 11. Impact of fasting period (3 or 5 wk) on *Trypanosoma cruzi* parasite load (± SEM) (33 days post-ingestion). (T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

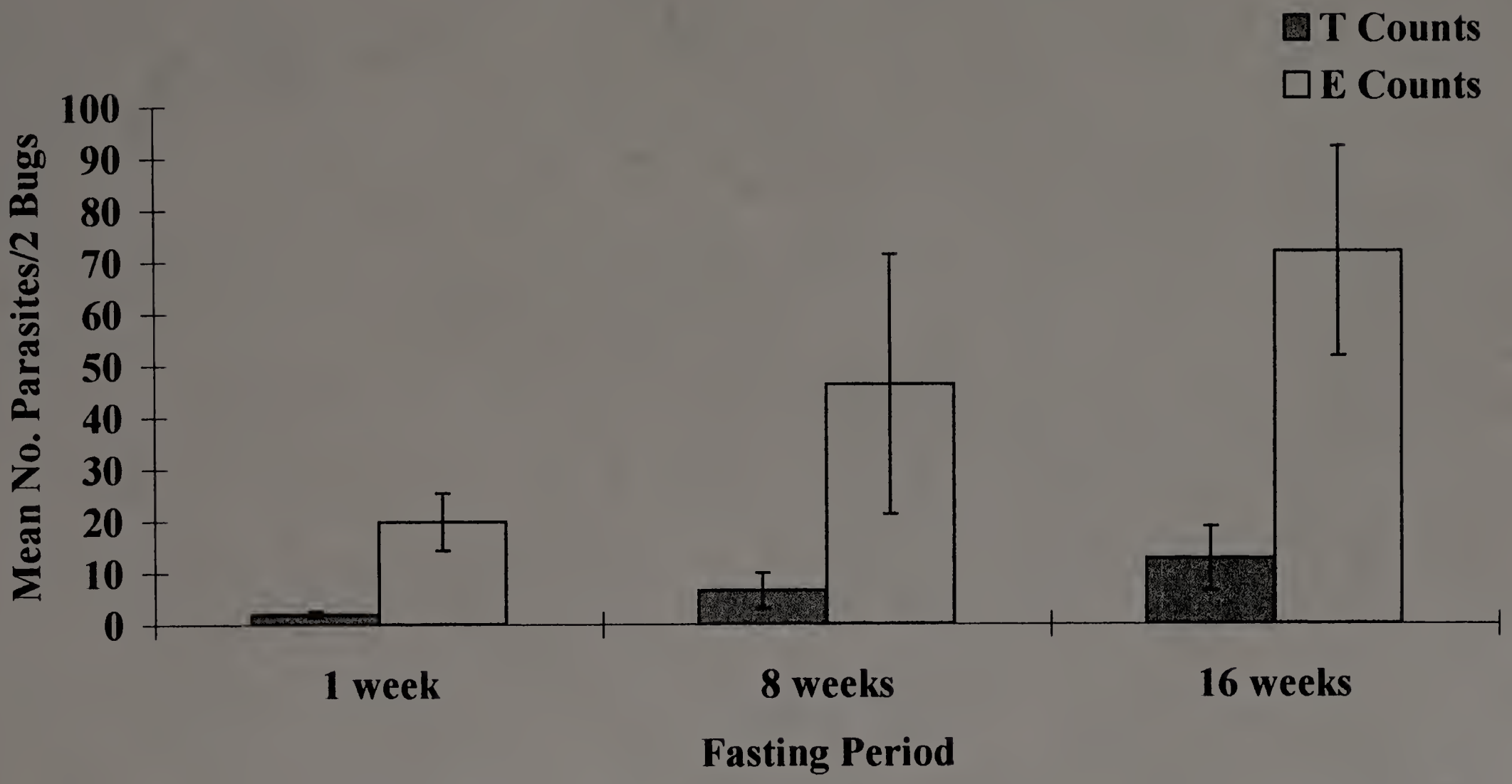

Figure 12. Impact of fasting period (1, 8, or 16 wk) on *Trypanosoma cruzi* parasite load (± SEM) (33 days post-ingestion). (T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

Discussion

TS remains catalytically active and detectable in the insect vector for 10-12 days. The addition of exogenous TS types does not appear to have any beneficial or deleterious impacts on the life history parameters of *R. prolixus* adults. Although the addition of rTS2000 increased *T. cruzi* parasite load, this genetic form of the enzyme is never seen in nature. In addition to lacking the carboxyl-terminus tandem repeats, epimastigote TS also differs from trypomastigote TS by being a monomeric enzyme (Schenkman et al., 1994; Briones et al., 1995).

The inconsistent results of adding exogenous TS types with *T. cruzi* might be explained if the population structure of the parasites had been more strictly controlled; the proportion of *T. cruzi* (Silvio strain) producing TS consists of 20-30% of the entire population. However, the results do suggest that TS is not the sole factor influencing *T. cruzi* parasite load in the insect, and that other elements are involved.

The addition of TS antibodies significantly increased the parasite load. Although the binding of TS antibodies to the enzyme does not inhibit TS activity, the act of binding to the enzyme apparently enhances the parasite's ability to multiply within the insect. However, conclusions regarding the mechanism of action cannot be drawn until experiments regarding the interaction of *T. cruzi* and TS at the histological level are performed. Preliminary transmission electron microscopy studies examining the gut of adult female *R. prolixus* demonstrated the presence of spherite-like particles in the posterior intestine, where they have never been recorded before. Spherites are thought to concentrate inorganic minerals. However, preliminary evidence did not suggest that either *T. cruzi* parasites or TS causes the histology of the gut to change in any way.

Rhodnius prolixus is quite resistant to starvation and the duration of fasting appears to have no predictable effect on *T. cruzi* parasite load. Perhaps the overall health of the insect or the level of particular digestive enzymes present in the insect is more important in regulating parasite load. The relationship between fasting and *T. cruzi* development remains unclear though parasite loads do not appear to be affected by prolonged starvation periods. This suggests that starvation is not a major obstacle for the parasite, especially since field insects may seldom experience the degree of starvation to which they were subjected these experiments.

CHAPTER IV

FRACTIONATION OF *TRYPANOSOMA CRUZI* SUBPOPULATIONS BY *TRANS*-SIALIDASE PHENOTYPE: IMPLICATIONS FOR PARASITE LOAD DEVELOPMENT IN *RHODNIUS PROLIXUS*

Introduction

The differential morphology (broad and slender) of trypomastigotes was first noted by Carlos Chagas (1909). However, the purpose of two different forms with regards to the parasite's life history has remained a mystery. Before the 1990's, researchers investigating the role of differential parasite morphology had no alternative except to work with strains predominated by either broad or slender forms. The work of Brener (1971) provided evidence that slender forms appeared to be incapable of developing within the insect vector, while broad forms were able to transform into epimastigotes.

Pereira et al. (1996) demonstrated that only a small subset (20-30%) of Silvio *T. cruzi* parasites phenotypically express TS (TS^+). In addition, TS^+ parasites can transform into TS^- parasites and vice versa. Pereira *et al.* (1996) were able to fractionate TS^+ and TS^- parasite subpopulations (Silvio strain) using magnetically-charged antibody beads, and discovered that TS^+ parasites were highly invasive of mammalian cells whereas TS^- parasites were only weakly invasive. Addition of exogenous TS to TS^- parasites increased invasiveness to levels indistinguishable from those of TS^+ parasites. Silvio strain TS^+ parasites are broad and slow-moving; TS^- parasites are very slender and move in a quick, snake-like motion. With the technology available to separate these two

morphologies, the development of fractionated *T. cruzi* subpopulations, separated on the basis of TS phenotype, was investigated within the insect vector.

Materials and Methods

Insects

Rhodnius prolixus was maintained in the laboratory at 28° C, 80% humidity, and a 14L:10D photoregime. Insects were separated by instar in large glass rearing jars and fed blood approximately every other week. Experimental insects were selected 2-3 weeks after a blood meal. Insects that took a blood meal and molted to the next stage were easily identified.

Fourth instars were starved for 3-5 weeks after molting before they were fed an infective blood meal containing *T. cruzi*. Parasite concentrations were adjusted so that the average insect would ingest approximately 7500 parasites within one replete blood meal. Insects failing to feed or to molt into fifth instars were discarded from the study.

Blood

Citrated (129 mM) rabbit blood was used as the blood meal in all experiments. Rabbit blood was purchased from a local vendor (Millbrook Farms), who collected rabbit blood from an ear vein and delivered it the next day in a vacutainer to the Medical Entomology Laboratory at the University of Massachusetts. The blood to be infected with *T. cruzi* was pooled into a larger tube and transported on ice to Tufts University.

Parasites

Three strains (Silvio, Montalvania-13, and Tulahuen) of *T. cruzi* trypomastigotes were obtained from Miercio Pereira of Tufts University. Parasites at Tufts are maintained at 37° C and passed through Vero cell cultures daily. Cultures containing trypomastigotes are washed once or twice with 10% FCS-RPMI and centrifuged at 1000 gravities x 10 min. Trypomastigotes were counted from the culture and an appropriate volume containing the desired number of parasites was added to the blood. Infected blood was packed on ice and transported back to the Medical Entomology Laboratory for immediate use in an experiment.

Trans-sialidase

All TS types were obtained from Tufts University. *Trypanosoma* TS was purified from trypomastigote cultures while the recombinant TS types (19y rTS and rTS2000) were produced by transformed *Escherichia coli* XL-1 containing TS inserts. Purification of recombinant forms was done in a manner identical to that of *Trypanosoma* TS, as briefly described in Chapter 3.

TS types were maintained on ice until incorporated into individual blood meals. Once the enzyme had been added to the blood, it was administered to the bugs as quickly as possible.

Trans-sialidase specific activity assay

TS activity was determined in the following reaction mixture combined in the respective order in a 1.5 ml microcentrifuge tube: 12 µl distilled water, 6 µl BSA

(10 mg/ml), 12 µl 50 mM Tris-HCl (pH = 7.2), 10 µl fetal calf serum (FCS), 20 µl bug gut extract, and 10 µl ^{14}C-lactose (40-50,000 cpm). Control tubes contained the same materials in equal volumes with the single exception that 20 µl of distilled water was added instead of 20 µl of bug extract. Once all of the materials had been added, the mixture was vortexed briefly and incubated at room temperature for approximately 21 hr. The reaction was stopped by the addition of 1 ml of water per reaction tube.

Each reaction mixture was transferred to an ion exchange reaction column containing a glass bead; the tip was loaded with the ion exchange resin (2 g QAE Sephadex A-50 in 200 ml distilled water) until a 1 ml column bed volume was formed.

The unbound components following the TS reaction were eluted with five aliquots of 1 ml distilled water. Bound TS components were eluted with 1 ml 1 M NaCl. Eluate was transferred to a scintillation vial containing 2 ml EcoScint scintillation fluid and shaken briefly. Vials were placed in a β-liquid scintillation counter and radioactivity determined.

Fractionation procedure

Parasites were fractionated with the use of mAb TCN-2 and dynabeads (Pereira et al., 1996). Successful fractionation of purified TS^+ and TS^- parasite populations was confirmed by assaying for TS activity by two methods: (1) microscopic examination and (2) assaying for TS activity. Parasite expressing TS, or TS^+ trypomastigotes, were broad and slow-moving while TS^- Silvio trypomastigotes, which lacked TS expression, were slender and moved very quickly; under microscopic examination, the level of fractionation

success could be estimated. Samples of each subpopulation were removed and measured for TS activity.

A successful fractionation was determined to be one in which the ratio TS activity of TS+ to TS- parasites was at least 4.5-fold higher. Fractionation success was dependent upon such factors as: (1) age and health of parasites, (2) age of antibodies used to fractionate the parasites, and (3) freshness of the cell culture.

Because parasites can transform from one phenotype to another after one hour, insects were fed immediately after parasites were isolated and incorporated into their respective blood meals. All groups were fed within 2 hr of each other and monitored for 33 days afterwards to record mortality and molting.

Experimental design

Blood meals, TS type (*Trypanosoma* vs. recombinant), enzyme activity levels, and 4th instar fasting periods were varied among a series of experiments. All parasite strains and TS were obtained from Miercio Pereira at Tufts University. For comparison with *Trypanosoma* TS, only 10% of recombinant TS enzyme (cpm/μl/hr) was used since the latter has a 10-fold higher protein content than the former.

All experiments in this chapter were followed according to a standardized procedure following blood meal preparation. Each blood meal contained enough parasite for an individual bug to ingest approximately 7500 trypomastigotes. Insects were allowed to feed on their blood meals for 30 minutes. Bugs failing to feed to repletion were discarded. Remaining insects were observed daily for 33 days to record mortality and molting. On day 33, insects were chilled and dissected live in phosphate buffered solution

(PBS): four hindgut/rectum samples were removed and homogenized in a 1.7 ml microcentrifuge tube. After live "wiggler" counts (metacyclic trypomastigotes + epimastigotes) were determined on a hemacytometer, parasite samples were further processed and purified for stained slide preparation (Diff-Quik®) to determine differential parasite counts.

Discrimination between metacyclic trypomastigote (metacycs) and epimastigote (epis) morphological forms was based on the location of the kinetoplast in relation to the nucleus. Parasite loads were quantitated by metacyclic trypomastigote (T count) and epimastigote (E count) infection levels. Data were analyzed with one-way ANOVAs and considered to be significant only below levels of $p < 0.05$.

$$\text{T count} = \text{wigglers} \times \frac{\text{metacycs}}{\text{metacycs + epis}} \qquad \text{E count} = \text{wigglers} \times \frac{\text{epimastigotes}}{\text{metacycs + epis}}$$

Ingestion of fractionated *Trypanosoma cruzi* subpopulations (Silvio strain)

Fourth instar *R. prolixus* (n = 50), starved for 4 weeks after molting, were allowed to feed on blood containing either fractionated TS-producing (TS^+), non-TS-producing (TS^-), unfractionated parasites or uninfected blood. All parasites were of the Silvio strain.

Ingestion of fractionated Silvio subpopulations with various *trans*-sialidase types added

Parasite subpopulations (TS^+ and TS^-) were incubated for 30 min with various types and activity levels of *trans*-sialidase: *Trypanosoma* TS (41,000 or 410,000 total

cpm), 19y rTS TS (bacterial recombinant) with 4,000 or 38,000 total cpm, or rTS2000 TS (bacterial recombinant lacking the carboxyl-terminus tandem repeats responsible for enzyme oligomerization) with 4,000 total cpm. The TS types were added to TS^- parasites to incubate before the enzyme was removed by centrifuging the parasites and removing excess enzyme. Parasites were then added to blood meals and ingested by bugs. Bugs were starved for 3-5 wk unless otherwise designated.

Rate of Silvio subpopulation development and interaction with TCN-2 antibodies

Starved 4th instars were fed Silvio strain *T. cruzi* subpopulations. The kinetics of unfractionated Silvio parasites interacting with TCN-2 antibodies was also studied simultaneously. Infected bugs (only those successfully molted to 5th instars) were assayed for *T. cruzi* infection by processing dissected, homogenized hindgut/rectum samples at the end of the experimental period (day 10, 20, 30, 45, and 60). Three guts/tube comprised one sample. Parasite loads were quantitated in the manner described earlier.

Interaction of fractionated Silvio subpopulations with TCN-2 antibodies

Starved 4th instars were given blood containing fractionated Silvio subpopulations, with or without TCN-2 at a concentration of 1.0 μg/ml blood. Control blood contained an IgG1 murine monoclonal antibody specific for spectrin. Fed insects were monitored for 33 days and parasite loads quantitated as described previously.

Ingestion of fractionated Tulahuen and Montalvania-13 strain subpopulations

Starved 4th instars were fed fractionated subpopulations of Tulahuen and Montalvania-13 strain *T. cruzi* parasites. Fed insects were again monitored for 33 days before parasite loads were quantitated.

Results

Ingestion of fractionated Silvio subpopulations

The fractionation procedure was successful; the TS activity levels of TS^+/TS^- parasites was 14.0, well beyond the 4.5-fold threshold level. Bugs ingesting fractionated TS^- parasites had significantly higher T counts and E counts than bugs ingesting fractionated TS^+ parasites or unfractionated parasites (Fig. 13).

Ingestion of fractionated Silvio subpopulations incubated with *Trypanosoma trans*-sialidase

The ratio of TS activity between TS^+ and TS^- parasites was 6.0-fold. The T counts among the four groups of bugs were not significantly different. However, the E counts were significantly higher in bugs ingesting TS^- parasites than in those ingesting TS^+ parasites or unfractionated parasites (Fig. 14). The group ingesting TS^- parasites incubated with total 410,000 cpm of *Trypanosoma* TS did not differ significantly from those ingesting only TS^+ or TS^- parasites but intermediate parasite loads suggest there may have been downregulation (Fig. 14).

Ingestion of fractionated Silvio subpopulations incubated with 19y rTS (starved 8 wk)

The ratio of TS activity between TS^+ and TS^- parasites was 13.3-fold. The T counts were significantly higher in groups ingesting TS^+ parasites and TS^- parasites than TS^- parasites incubated with 19y rTS for 30 min (Fig. 15). However, only bugs ingesting TS^- parasites had a significantly higher T count than unfractionated parasites (Fig. 15). Both bug groups ingesting TS^+ and TS^- parasites had significantly higher E counts than groups ingesting TS^- parasites incubated with 19y rTS or unfractionated parasites (Fig. 15).

The fact that the TS^+ parasites produced a higher relative parasite load to TS^- parasites than in the previous experiment can probably be explained by the fact that the TS activity of unfractionated parasites was higher than that of TS^+ parasites, strongly suggesting that the TS^+ parasites were contaminated with TS^- parasites. Any addition of TS^- parasites, as suggested by my data, would help to increase the parasite load. The possibility also exists that the slightly longer fasting period of 8 wk may have caused gut changes affecting the kinetics of parasite subpopulation growth, by shifting the balance between various gut enzymes or nutrients.

Ingestion of fractionated Silvio subpopulations incubated with 19y rTS (starved 2-3 wk)

The ratio of TS activity between TS^+ and TS^- parasites was 13.3-fold. Insects ingesting TS^- parasites had significantly higher T counts than the other three groups (Fig. 16). There were no significant differences in E counts, probably due to variability

and very small sample sizes (Fig. 16), as well as the probably contamination of TS^+ parasites with TS^- parasites.

Ingestion of fractionated Silvio subpopulations incubated with various *trans*-sialidase types

A ratio of 28-fold was observed between the TS activity of TS^+ and TS^- parasites. The E counts were significantly higher in bugs ingesting TS^- parasites, than those ingesting TS^+ parasites or TS^- parasites incubated with either low and high levels of *Trypanosoma* TS (41,2000 and 412,000 total cpm, respectively) or 19y rTS (4,000 total cpm) (Fig. 17). All E counts of these groups were significantly higher than bugs ingesting rTS2000 (4,400 total cpm) or unfractionated parasites (Fig. 17).

The T counts were significantly higher in groups ingesting TS^+, TS^-, TS^- parasites incubated with high *Trypanosoma* TS and 19y rTS (4,000 total cpm) than those ingesting TS^- parasites incubated with rTS2000 or unfractionated parasites (Fig. 17). The T counts were significantly higher in those bugs ingesting low levels of *Trypanosoma* TS (4,000 total cpm) than in those ingesting unfractionated parasites (Fig. 17).

Rate of Silvio subpopulation development and interaction with TCN-2 antibodies

The fractionation was successful and produced a ratio of TS activity between TS^+ and TS^- parasites of 4.5-fold. These experiments were simply for observational purposes and were not statistically analyzed. Sample sizes usually consisted of two samples (6 bugs each) per time point, except for the occasional time at which only one sample (3 bugs) was available.

Overall, wiggler levels appeared to reach a maximum at 30 days post-infection (Figs. 18-21). The only exception is found in the TCN-2 experiment, which did not produce a maximal parasite load at day 30 (Fig. 21); sample variation was unusually high for this group (as compared to previous experiments). However, three days later, two more samples of the same group yielded 220 and 152 wigglers, suggesting that the low data point was spurious.

As expected, there appeared to be a general increase in the proportion of metacyclics, and they plateaued at 30 days (Figs. 18-21). However, among bugs ingesting TS^+ parasites, metacyclics continued to increase up to day 60 (Fig. 18). This suggests delayed differentiation or growth of parasites.

TS- parasites had the highest wiggler levels among all groups, over all time points except day 60 (Tables 5-7); at this particular time point, two of three samples had very low parasite loads. Perhaps a larger sample size would not have yielded such a low overall parasite load.

Interaction of fractionated Silvio subpopulations with TCN-2 antibodies

The number of wigglers, metacyclic trypomastigotes and epimastigotes of bugs ingesting TS^+ and TCN-2 was significantly higher than those ingesting only TS^+ (Fig. 22). There were no significant differences, however, between bugs ingesting TS^- parasites alone or TS^- parasites with TCN-2 (Fig. 22). The sample size of bugs ingesting unfractionated parasites with TCN-2 was too small to detect significant differences from those bugs ingesting only unfractionated parasites. The above results, however, are strongly suggestive and have been found statistically significant in four or five previous

experiments. Proportions of metacyclics vs. epimastigotes did not differ significantly between any paired group ($p > 0.05$).

Ingestion of fractionated Tulahuen and Montalvania-13 strain subpopulations

Dissections took place 30 days post-infection, 3 days prior to the final dissections to ascertain total parasite loads between experimental groups, to determine the proportion of individual insects infected with either Tulahuen (Tula) or Montalvania-13 (MV-13) strain. One hindgut was dissected and homogenized with 75 µl of 10% FCS-RPMI containing antibiotic. Ten bugs were dissected per group. The mean number of wiggler parasites for the Tula strain was 80.3 $\pm$ 10.2 parasites while that of the MV-13 strain was 141.1 $\pm$ 31.5 parasites. The Silvio strain infected my laboratory colony at a rate of 100%, producing comparable parasite loads to the Tula strain. The remaining insects were then dissected on day 33 to compare parasite loads of pooled and homogenized bug guts, following the protocol used for all previous infection experiments.

Despite what appeared to be a successful fractionation of Tula parasites, wiggler counts did not significantly differ between the groups. However, the parasite loads of bugs ingesting TS^- parasites was slightly higher than those of bugs ingesting TS^+ parasites or unfractionated populations (Fig. 23).

Although the first fractionation of the MV-13 strain was not good (TS activity of TS^+/TS^- parasites was 1.6-fold), bugs ingesting MV-13 TS^- parasites had a significantly higher parasite load than those ingesting MV-13 TS^+ parasites (Fig. 24). However, neither group significantly differed from the control (Fig. 24). There were no significant differences in proportions of metacyclic trypomastigotes or epimastigotes between groups.

A second fractionation proved to be more successful (TS activity ratio of TS^+/TS^- parasites was 3.3-fold). Wiggler counts for bugs ingesting only TS^- parasites were significantly higher than bugs ingesting either TS^+ parasites or unfractionated parasites (Fig. 25). The same pattern was followed for the E counts of bugs ingesting TS^- parasites (Fig. 25). However, the T counts of bugs ingesting TS^- parasites were significantly higher only than those bugs ingesting unfractionated parasites (Fig. 25). There were no significant differences in the proportions of metacyclic trypomastigotes or epimastigotes between groups ($p > 0.05$).

Mortality and molting activity

Mortality and molting activity did not significantly differ among 4th instars ingesting various fractionated subpopulations ($p > 0.05$) (data not shown). This supports the finding from my previous experiments (Chapter 3) which demonstrated that the ingestion of TS did not significantly impact the life history parameters of nymphs or adult females.

Table 5. Rate of *Trypanosoma cruzi* wiggler (trypomastigotes + epimastigotes) development (± SEM) in *Rhodnius prolixus*.

	Days Post-Ingestion				
Blood Meal	10	20	30	45	60
TS^+ Parasites	23.0 ± 8.0	33.0 ± 0.0	105.5 ± 3.5	85.7 ± 6.9	87.3 ± 8.7
TS^- Parasites	74.5 ± 35.5	196.5 ± 33.5	229.5 ± 42.5	155.0 ± 16.0	54.0 ± 25.3
Unfractionated Parasites	16.3 ± 9.8	80.5 ± 16.5	140.5 ± 11.5	120.5 ± 5.5	98.0 ± 5.5
Unfractionated Parasites with TCN-2 (1.0 µg/ml blood)	57.0 ± 11.0	152.0	115.0 ± 72.5	106.3 + 44.7	129.0 ± 2.0

Table 6. Rate of *Trypanosoma cruzi* trypomastigote development (± SEM) in *Rhodnius prolixus*.

	Days Post-Ingestion				
Blood Meal	10	20	30	45	60
TS^+ Parasites	1.0 ± 0.6	3.5 ± 0.8	8.5 ± 2.4	13.8 ± 3.5	14.8 ± 1.3
TS^- Parasites	2.1 ± 0.1	26.1 ± 13.0	48.0 ± 25.5	34.4 ± 13.5	12.2 ± 10.5
Unfractionated Parasites	2.0 ± 1.6	10.0 ± 0.3	25.6 ± 7.7	25.1 ± 10.2	12.4 ± 3.1
Unfractionated Parasites with TCN-2 (1.0 μg/ml blood)	2.9 ± 0.8	13.68	19.2 ± 14.5	10.0 + 2.0	19.3 ± 4.9

Table 7. Rate of *Trypanosoma cruzi* epimastigote development (± SEM) in *Rhodnius prolixus.*

	Days Post-Ingestion				
Blood Meal	10	20	30	45	60
TS^+ Parasites	22.0 ± 7.4	29.5 ± 0.8	97.0 ± 1.1	71.8 ± 3.5	72.5 ± 7.5
TS^- Parasites	72.4 ± 35.4	170.4 ± 20.5	181.6 ± 17.0	120.6 ± 2.5	41.8 ± 34.7
Unfractionated Parasites	14.3 ± 8.1	70.5 ± 16.8	114.6 ± 19.2	95.4 ± 4.7	85.6 ± 2.4
Unfractionated Parasites with TCN-2 (1.0 μg/ml blood)	109.7 ± 6.9	138.32	95.8 ± 58.0	96.3 + 42.6	109.7 ± 6.9

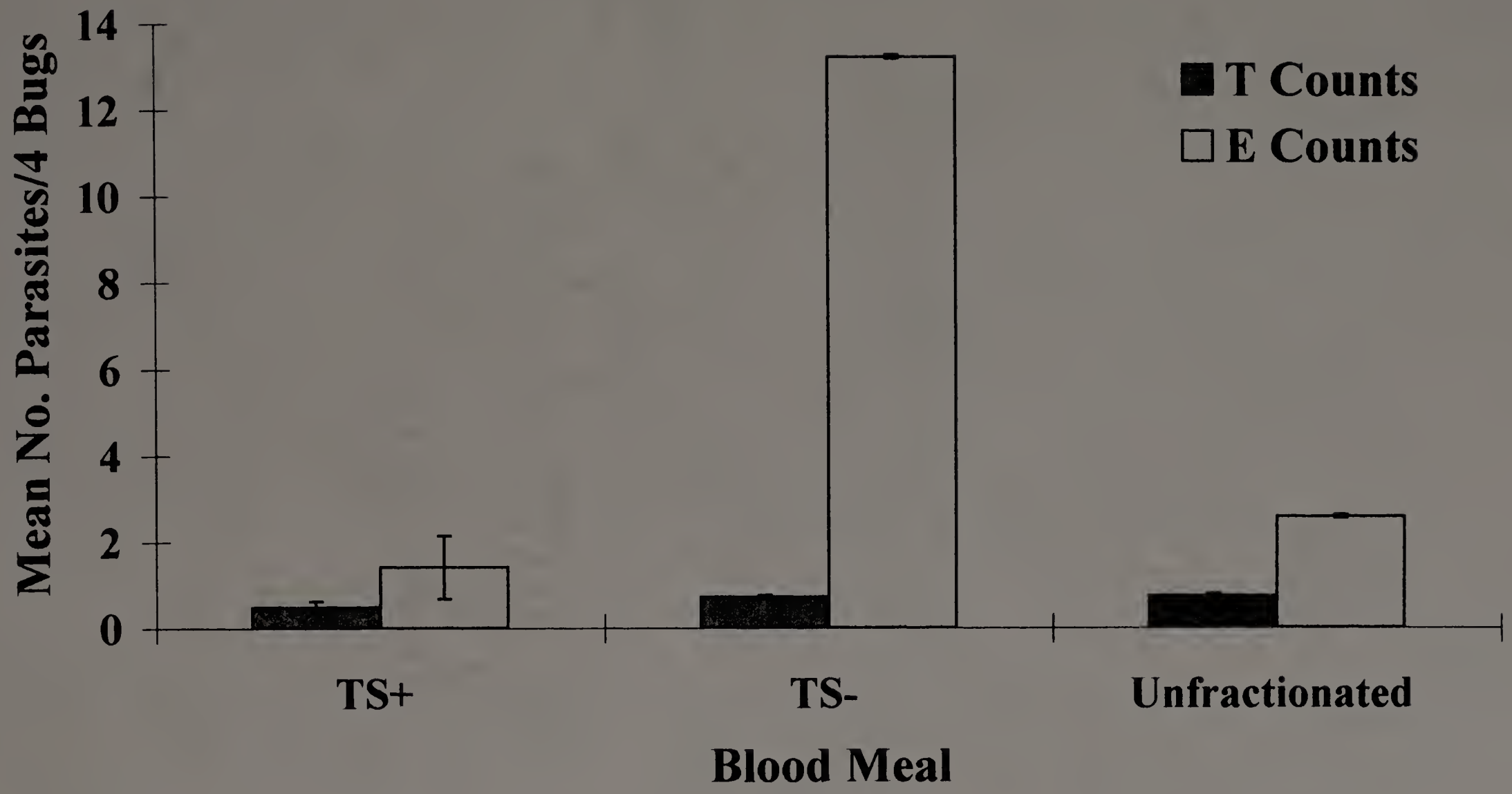

Figure 13. Parasite loads (± SEM) of fractionated *Trypanosoma cruzi* subpopulations in *Rhodnius prolixus* (33 days post-ingestion). (T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

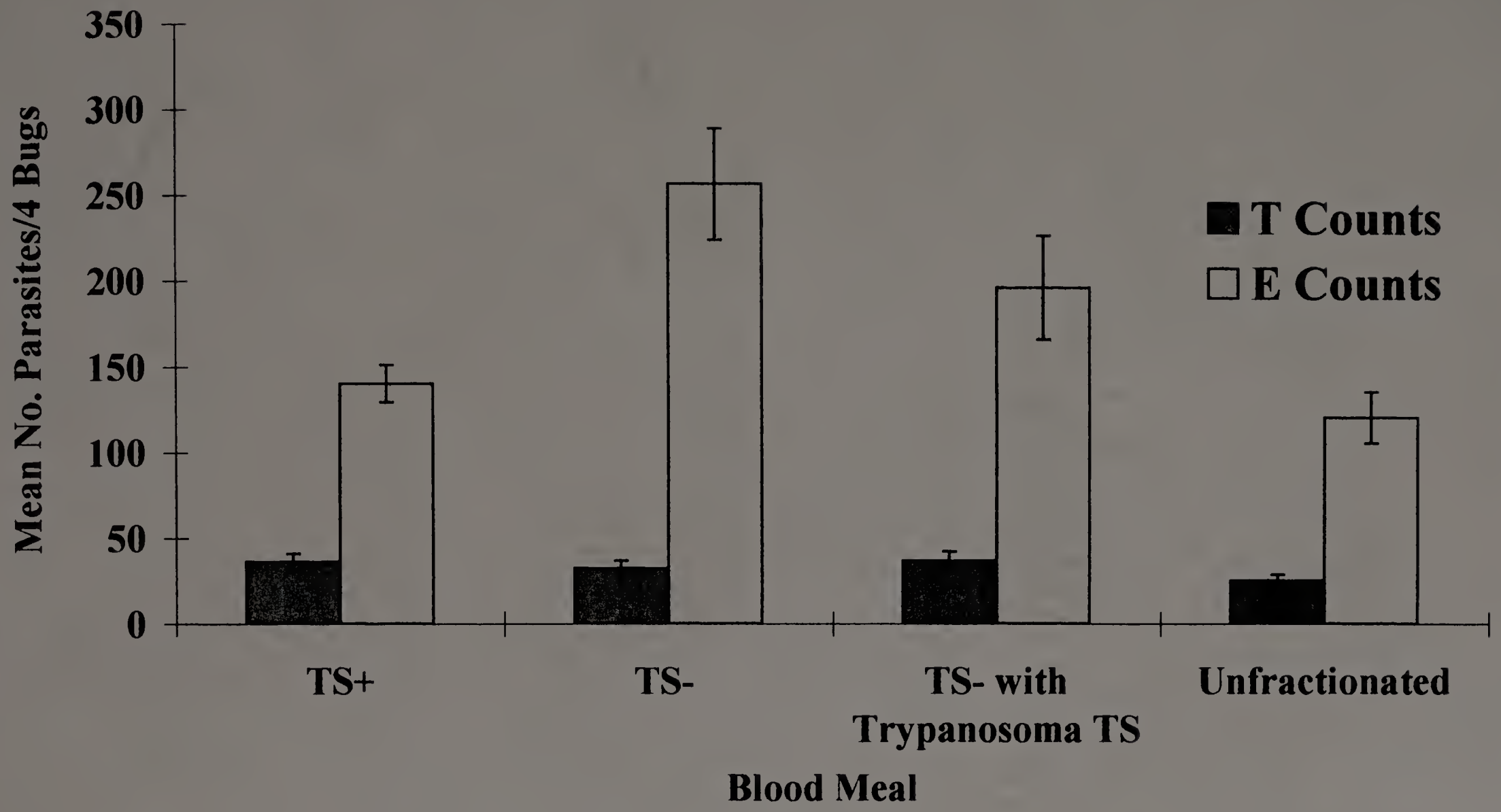

Figure 14. Parasite loads (± SEM) of fractionated *Trypanosoma cruzi* subpopulations in *Rhodnius prolixus* (TS- parasites incubated with *Trypanosoma trans* -sialidase) (33 days post-ingestion). (T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

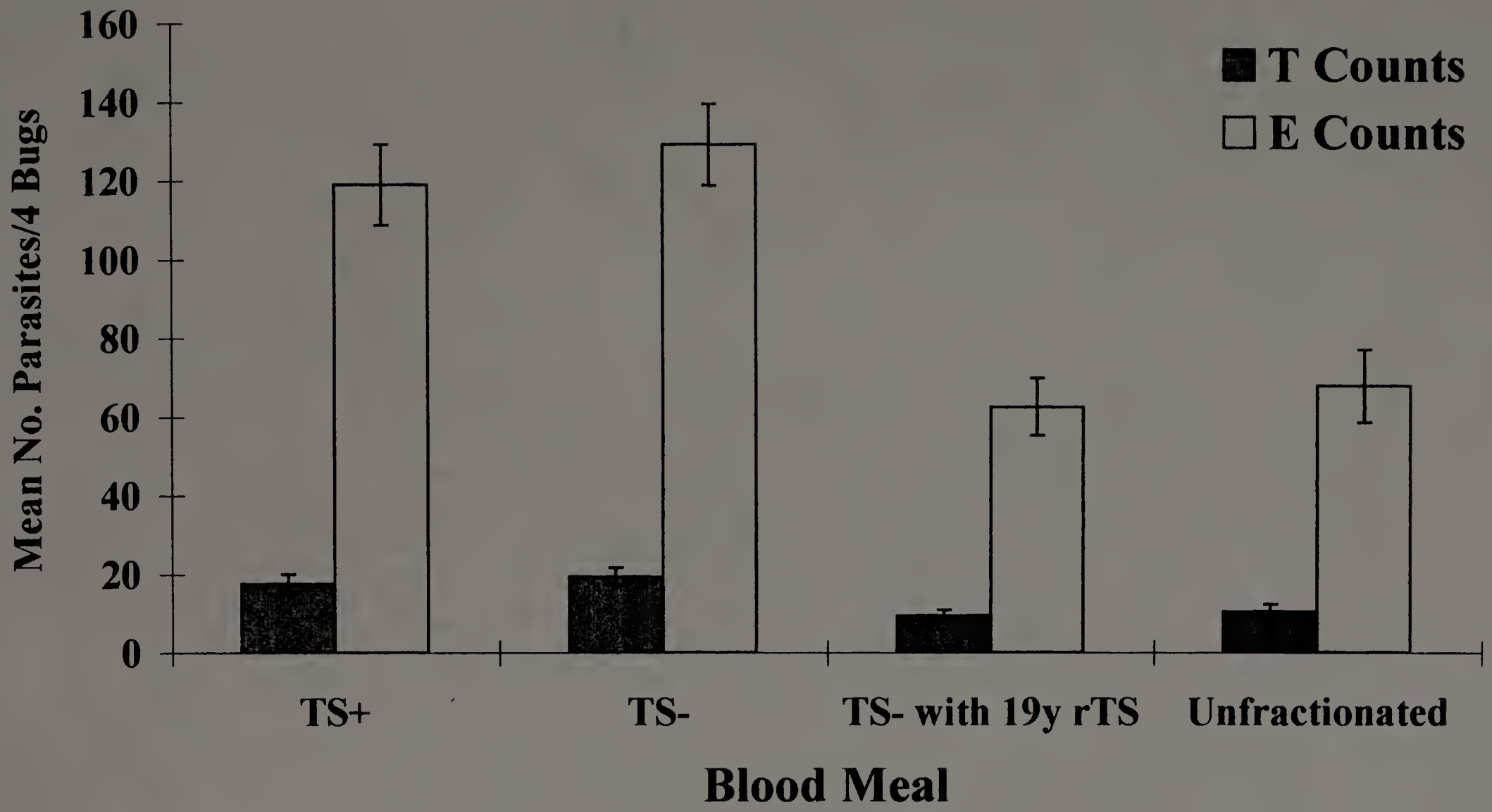

Figure 15. Parasite loads (± SEM) of fractionated *Trypanosoma cruzi* subpopulations in *Rhodnius prolixus*, starved 8 weeks (TS- parasites incubated with 19y rTS *trans*-sialidase) (33 days post-ingestion).
(T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

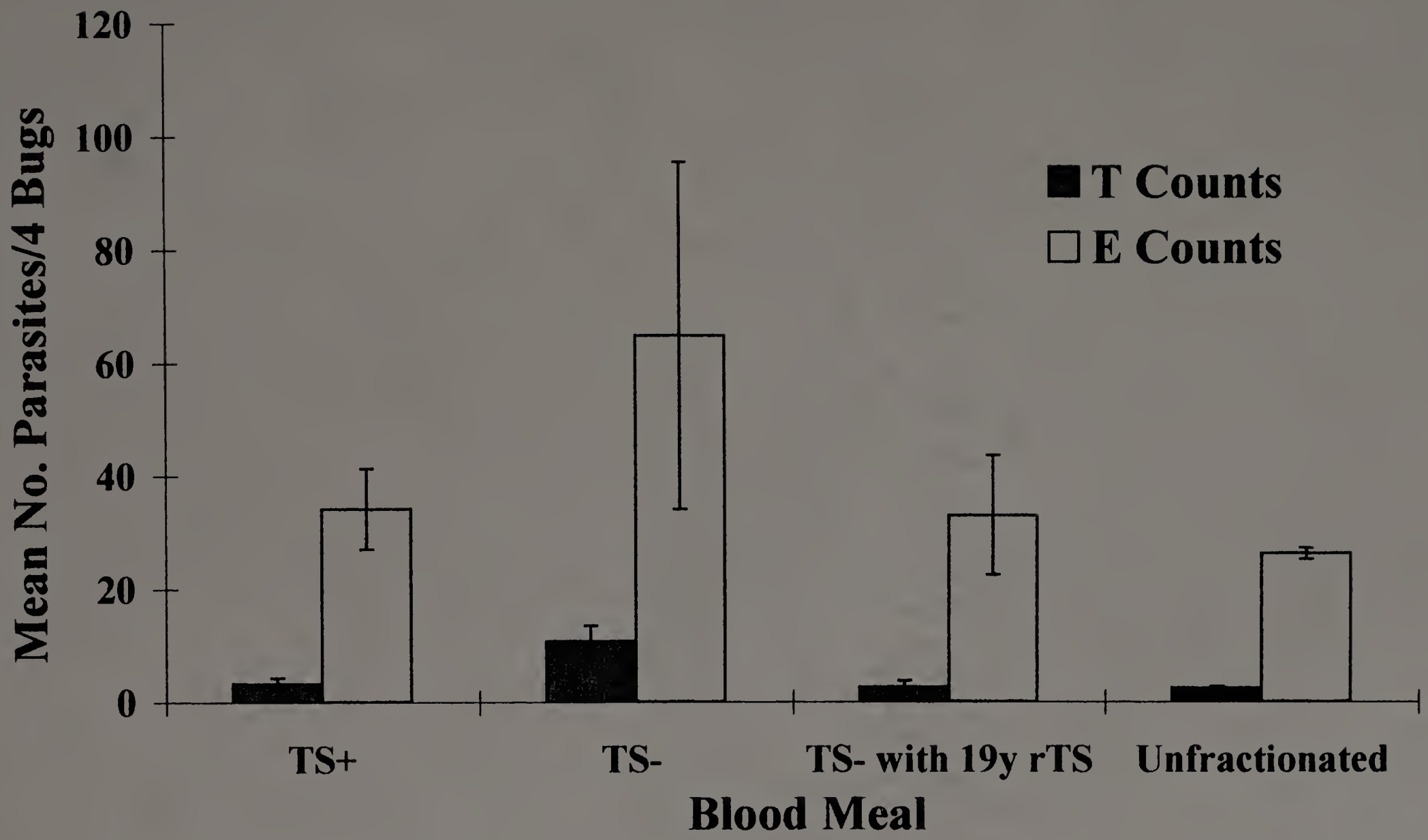

Figure 16. Parasite loads (± SEM) of fractionated *Trypanosoma cruzi* subpopulations in *Rhodnius prolixus*, starved 2-3 weeks (TS- parasites incubated with 19y rTS *trans*-sialidase) (33 days post-ingestion). (T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

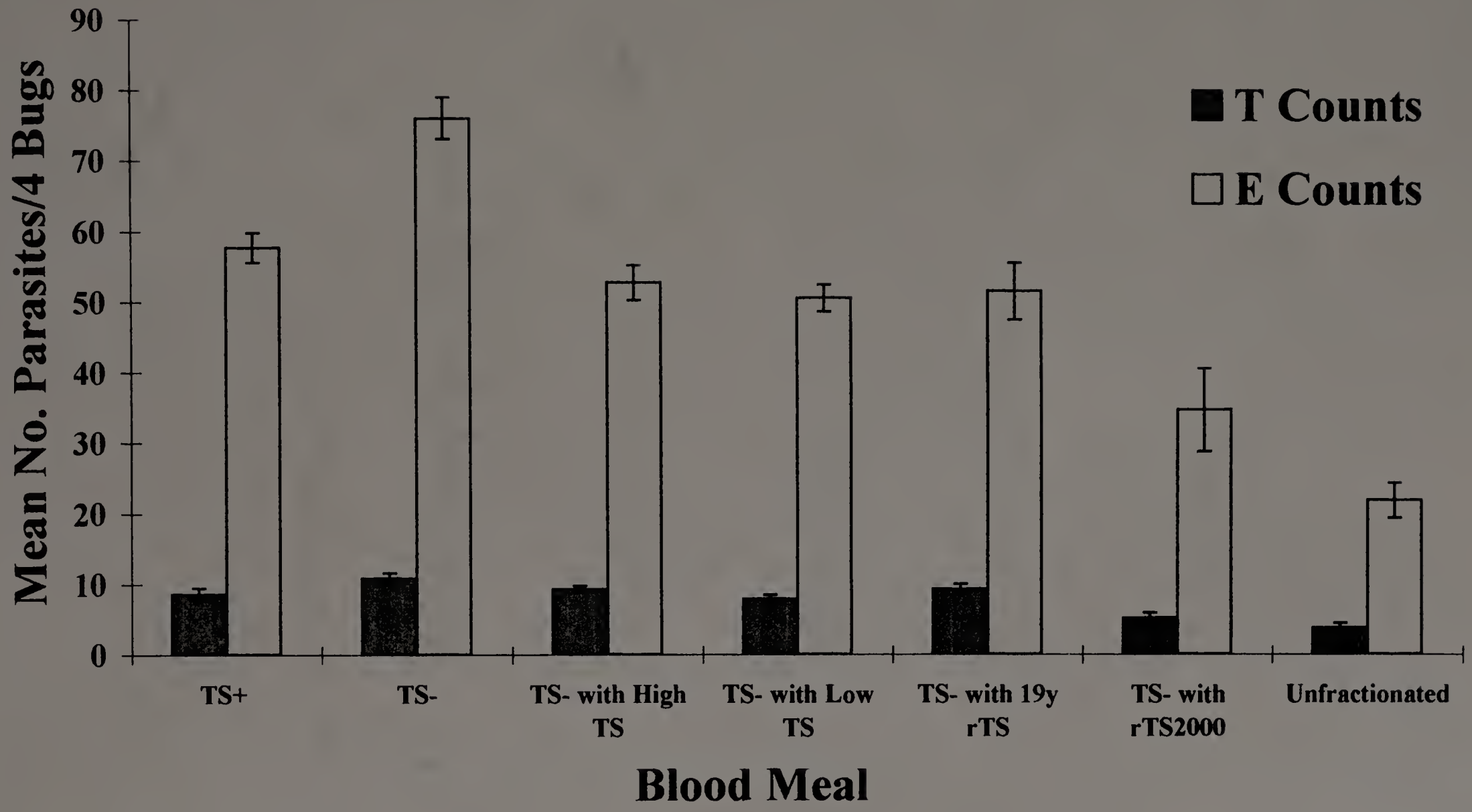

Figure 17. Parasite loads (± SEM) of fractionated *Trypanosoma cruzi* subpopulations in *Rhodnius prolixus* (TS- parasites incubated with various *trans*- sialidase types) (33 days post-ingestion). (T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

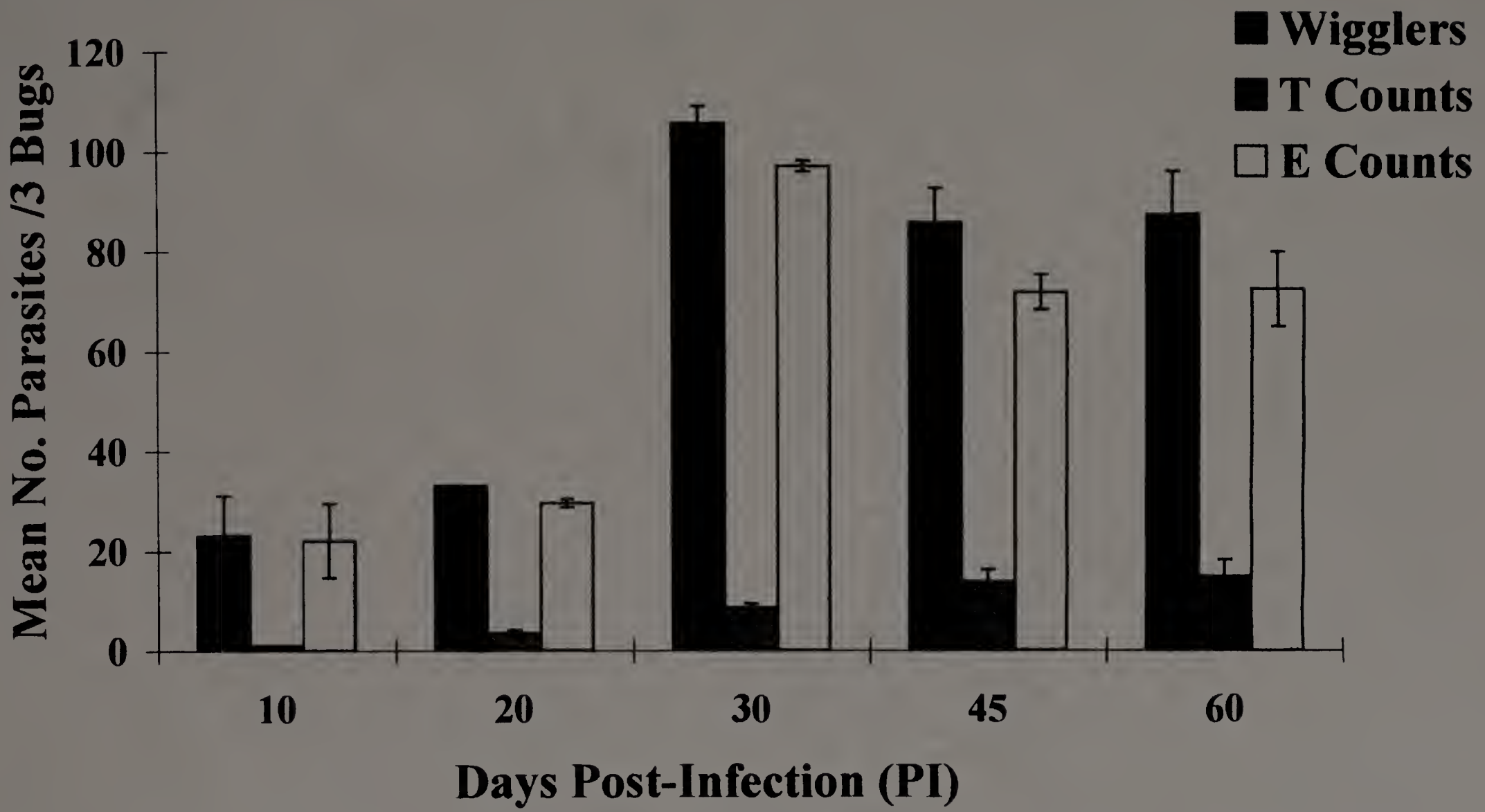

Figure 18. Parasite loads (± SEM) over time of fractionated TS+ parasites in *Rhodnius prolixus* (33 days post-ingestion). (Wigglers, total no. of trypomastigotes and epimastigotes; T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

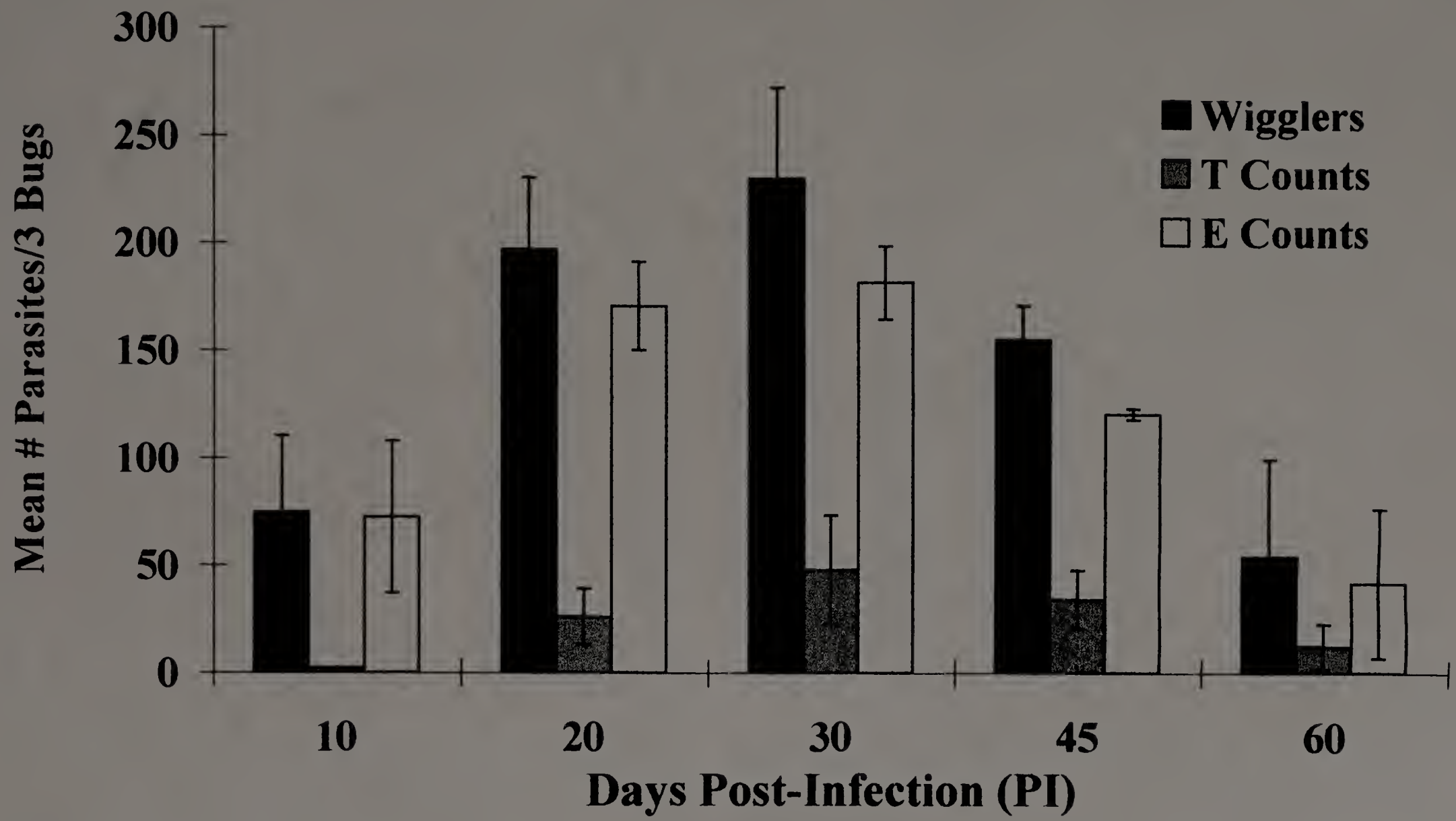

Figure 19. Parasite loads (± SEM) over time of fractionated TS- parasites in *Rhodnius prolixus* (33 days post-ingestion). (Wigglers, total no. of trypomastigotes and epimastigotes; T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

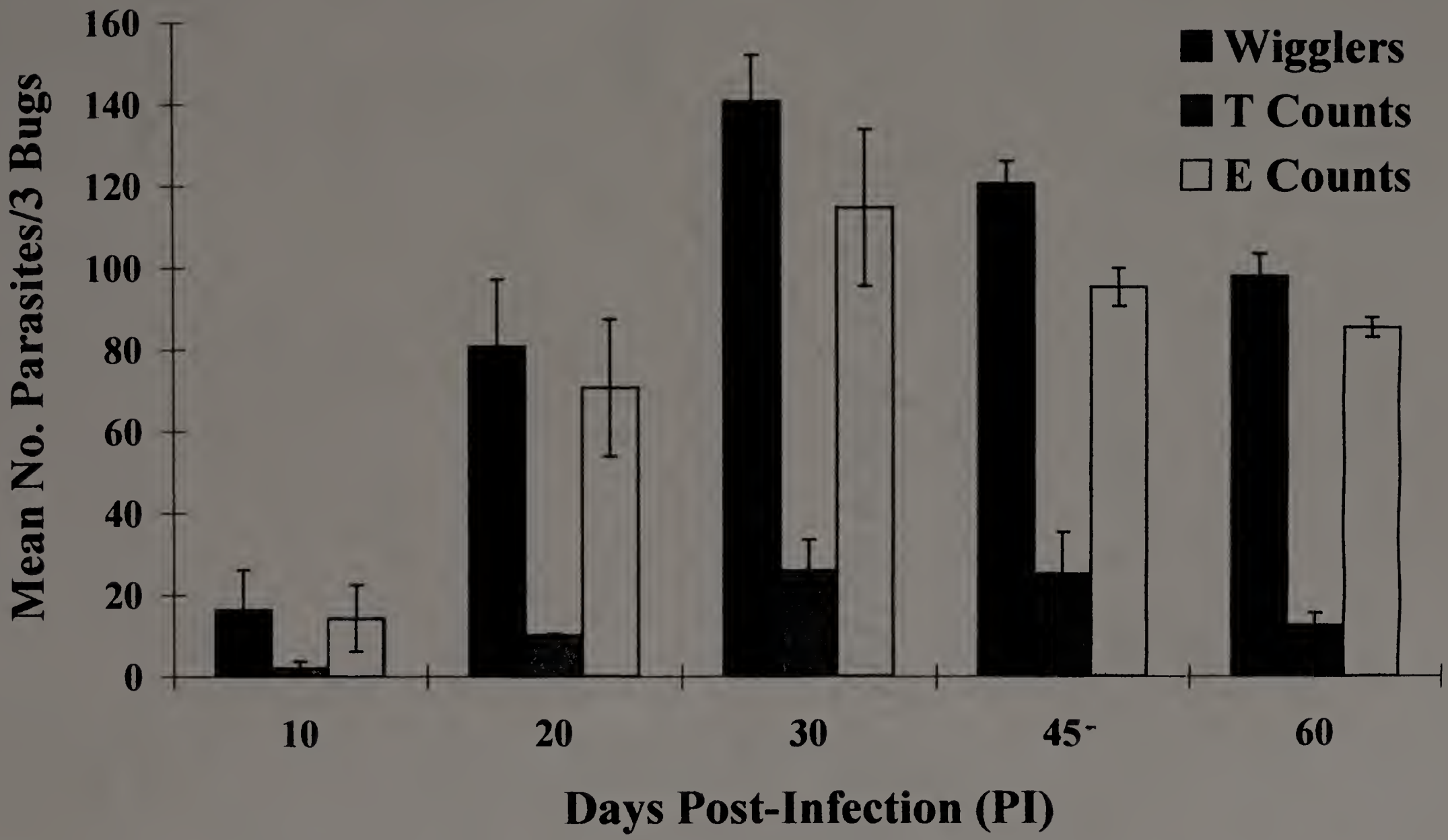

Figure 20. Parasite loads (± SEM) over time of unfractionated parasites in *Rhodnius prolixus* (33 days post-ingestion). (Wigglers, total no. of trypomastigotes and epimastigotes; T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

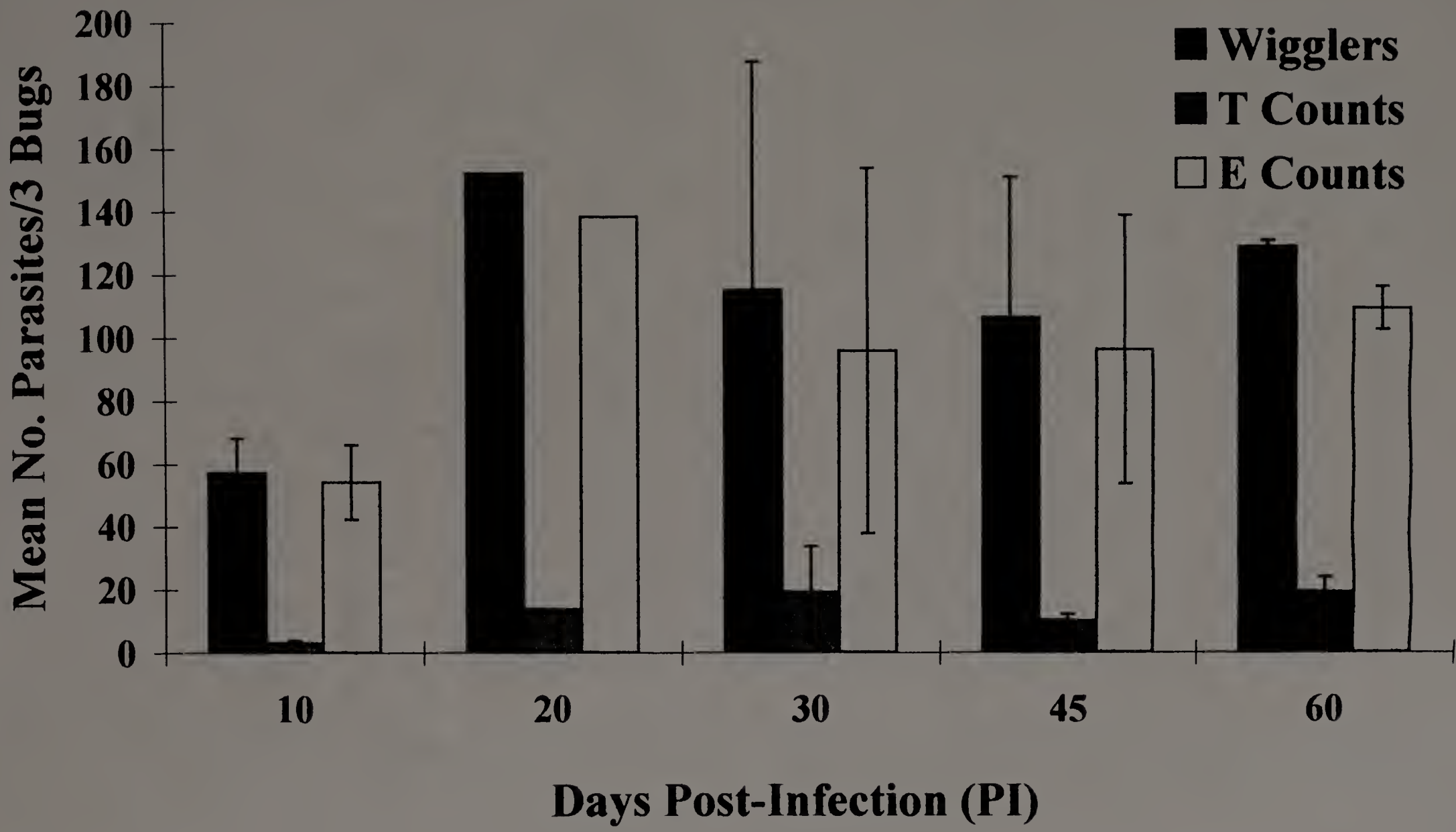

Figure 21. ***Trypanosoma cruzi*** **+ TCN-2 (1.0 µg mAb/ml blood) parasite loads (± SEM) over time in** ***Rhodnius prolixus*** **(33 days post-ingestion). (Wigglers, total no. of trypomastigotes and epimasigotes; T counts, no. of trypomastigotes; E counts, no. of epimastigotes)**

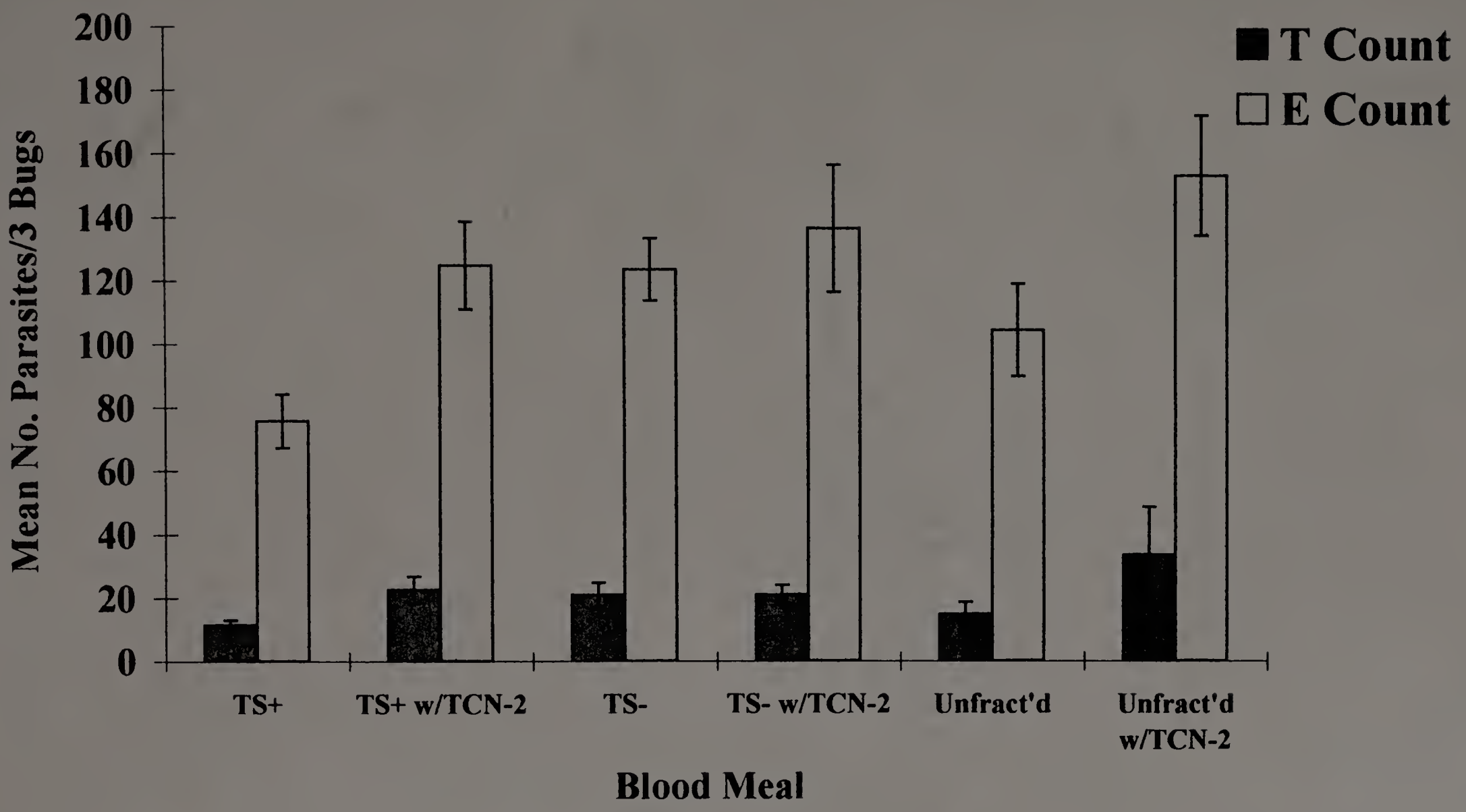

Figure 22. Impact of TCN-2 (1.0 µg mAb/ml blood) on parasite loads (± SEM) of fractionated *Trypanosoma cruzi* (Silvio strain) subpopulations in *Rhodnius prolixus* (33 days post-ingestion). (T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

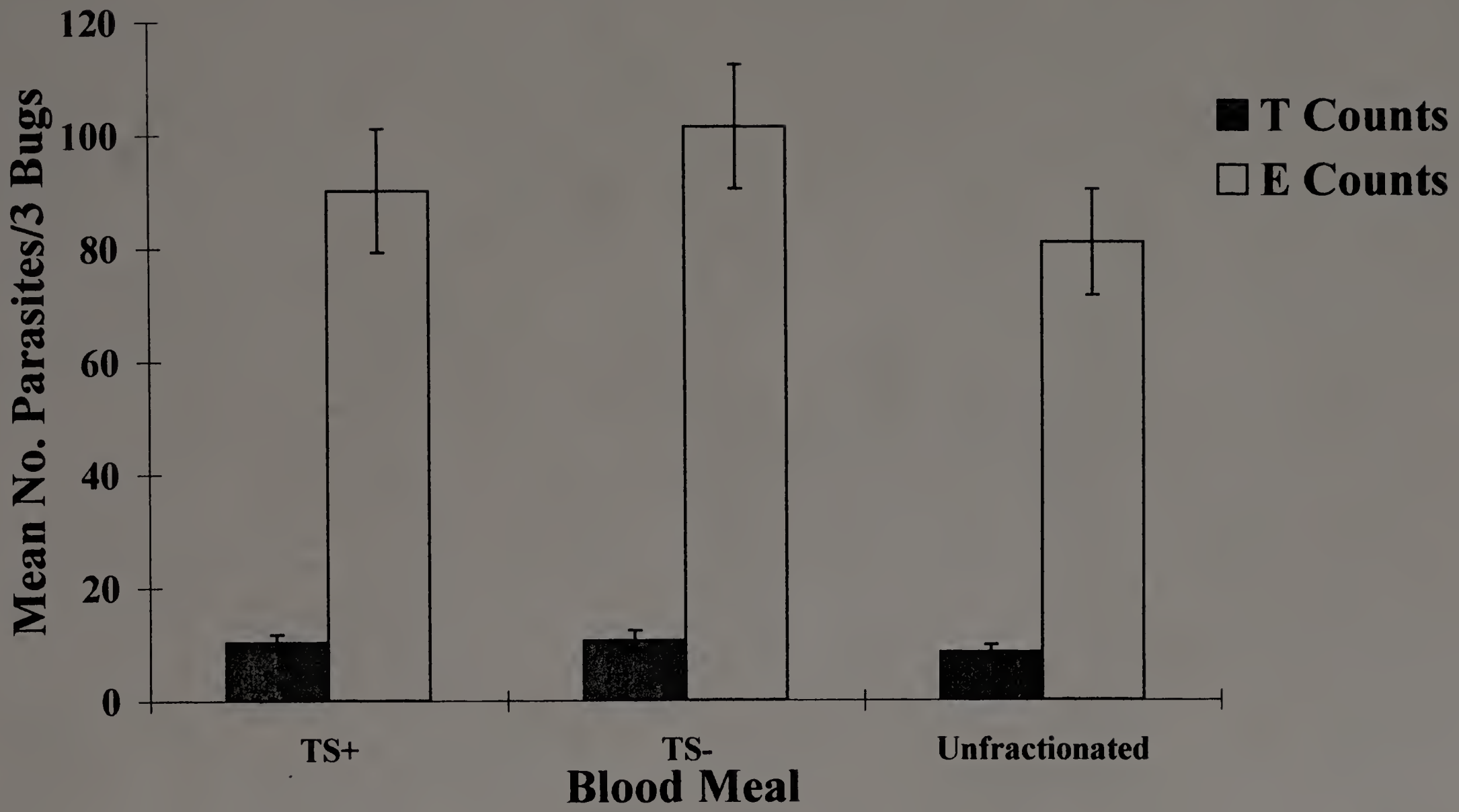

Figure 23. Parasite loads (± SEM) of fractionated *Trypanosoma cruzi* (Tulahuen strain) subpopulations in *Rhodnius prolixus* (33 days post-ingestion). (T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

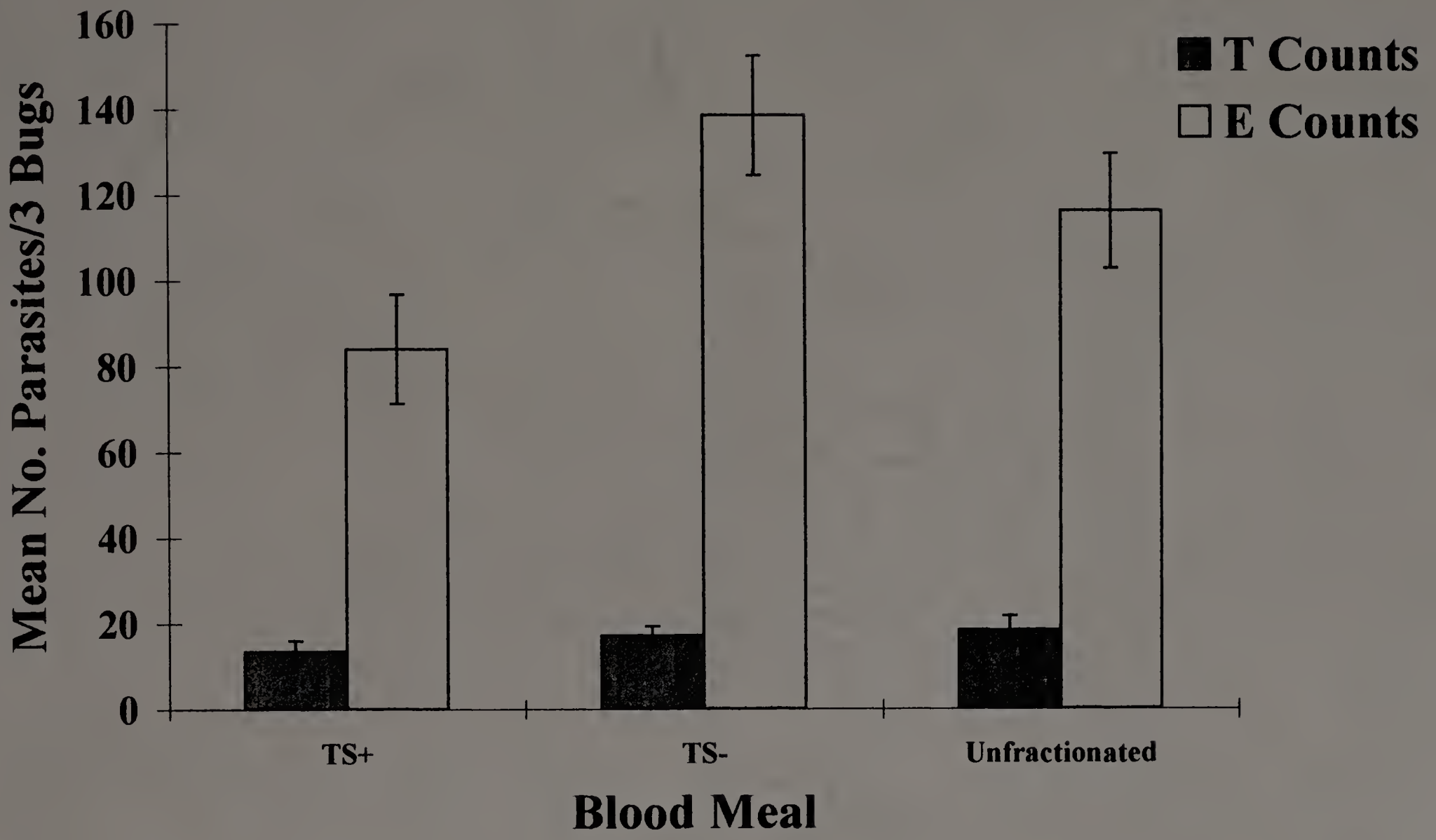

Figure 24. Parasite loads (± SEM) of fractionated *Trypanosoma cruzi* (Montalvania-13 strain) subpopulations in *Rhodnius prolixus* (33 days post-ingestion) [1st fractionation].
(T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

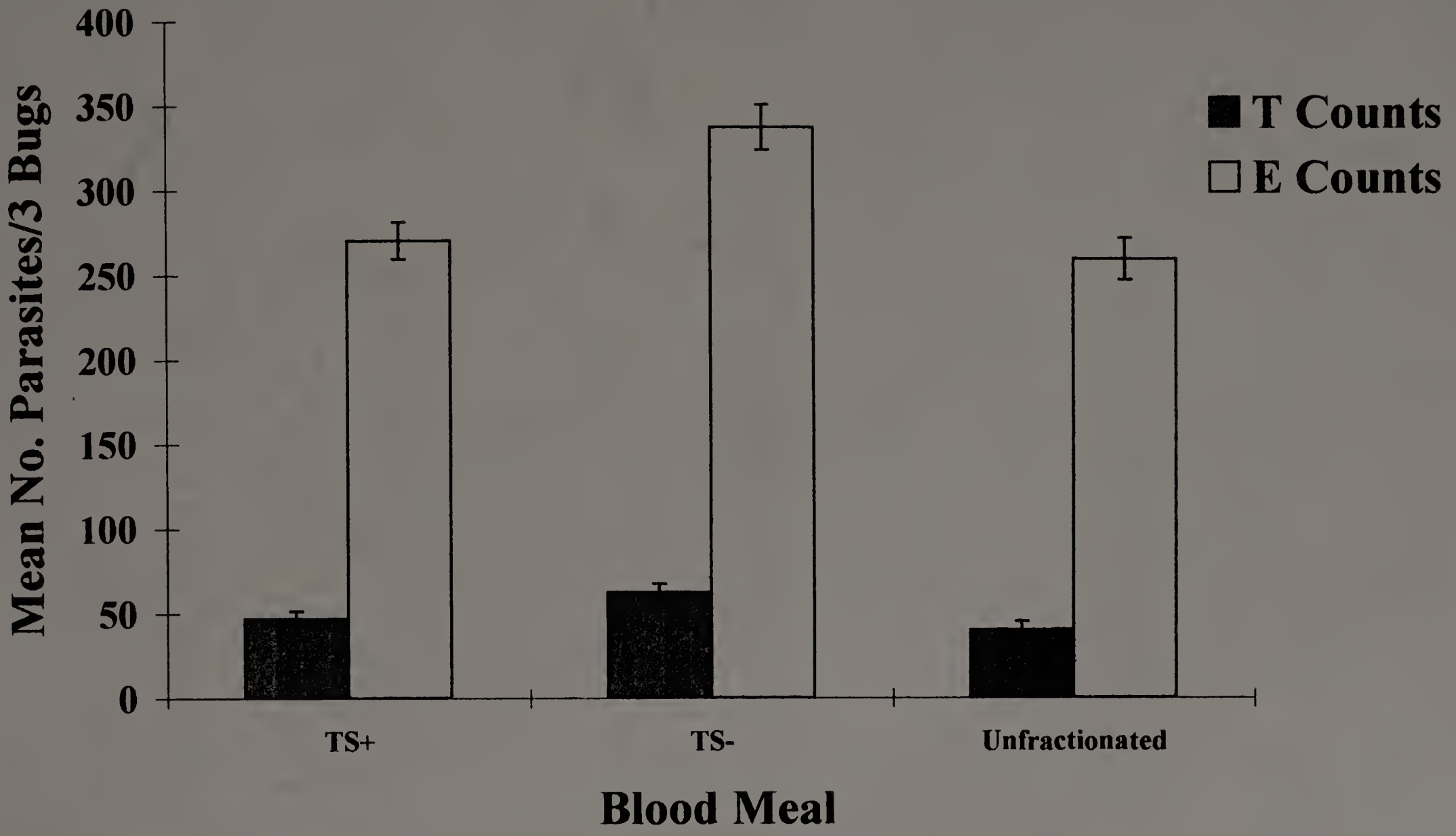

Figure 25. Parasite loads (± SEM) of fractionated *Trypanosoma cruzi* (Montalvania-13 strain) subpopulations in *Rhodnius prolixus* (33 days post-ingestion) [2nd fractionation]. (T counts, no. of trypomastigotes; E counts, no. of epimastigotes)

Discussion

My data indicate that ingested purified TS^- parasite populations increase parasite loads within *R. prolixus*, at least those of epimastigotes. Bugs feeding on a blood meal of TS^- parasites incubated with exogenous *Trypanosoma* TS, 19y rTS, or rTS2000 also had lower parasite loads, suggesting that the addition of TS reduces parasite loads in the insect vector.

Insects ingesting purified TS^- subset populations always had significantly higher parasite loads (E counts) than those ingesting unfractionated parasites. One possible explanation for this occurrence could be that TS^- and TS^+ parasite populations interact with each other, acting to regulate and control the parasite load. Too many parasites could kill the insect vector while too few parasites might significantly decrease chances of successfully infecting a mammalian host upon exit from the vector, either as a result of sheer lack of parasite numbers or an insufficient number of TS^+ phenotypic parasites producing and shedding TS. Another explanation is that the TS^+ phenotype is only important for mammalian cell attachment, penetration and exit and plays no role in the development of the parasite in the invertebrate vector. This hypothesis supports the evidence found by Amino et al. (1995), who suggested that *T. cruzi* did not need sialylation in order to survive in the gut of *T. infestans*. Because *T. cruzi* has been found to affect the production of amino acids in kissing bugs, perhaps it is not unreasonable to hypothesize that TS^- parasites are better able to acquire important metabolites than TS^+ parasites. However, it also seems entirely possible that TS^+ parasites produce a protein of some kind, which hinders their ability to invade and reproduce in the insect vector. Future studies must examine if other phenotypic differences exist between the two forms.

Inconsistencies in molting periods over the course of these experiments indicate that neither TS^+ nor TS^- subset populations appear to significantly influence the period of time required for 4th instars to molt. Mortality does not appear to be affected by ingested parasite subpopulations, either with or without the addition of exogenous TS types.

Although the data indicate a different biological role for TS^+ and TS^- subpopulations in the insect vector than in the mammalian system, this difference may represent survival tactics uniquely specialized for different environments, as is found with *Trypanosoma brucei* in mammals versus the tsetse flies. *Trypanosoma brucei* also expresses TS, though only in its insect form. Pontes de Carvalho et al. (1993) suggested two possible hypotheses: (1) TS may facilitate *T. brucei* interaction with fly's internal anatomy or (2) perhaps the sialylation of procyclin protects the parasite from attack of the host complement components still active within the blood meal. However, Tomlinson et al. (1994) discovered that sialylation was not the only mechanism by which *T. cruzi* trypomastigotes could resist lysis by the complement cascade. There may be a fitness cost associated with being a TS^+ parasite in the insect vector, though it appears to be a fitness advantage in the mammalian system.

My results suggest that TS^+ and TS^- phenotypes have different roles in the insect vector than the mammalian system. TS^+ and TS^- parasites may be interacting with each other, regulating parasite load in the insect vector. The fact that TS^- parasites develop more successfully in the insect vector suggests that the existence of two morphological forms of *T. cruzi* may have evolved as a mechanism for the parasite to invade two very different systems. TS^- parasites appear to be predisposed to enter and reproduce in the insect vector while TS^+ parasites are much better suited for invasion mammalian cells. By

producing heterogenous populations of parasites, *T. cruzi* increases its chances to infiltrate and develop within alternating hosts.

CHAPTER V

IMPACT OF *TRYPANOSOMA CRUZI* INFECTION ON *RHODNIUS PROLIXUS* BEHAVIOR

Introduction

In general, the most successful blood-feeding insects are those that can feed quickly on their host and escape detection (Gillett, 1966). There are several examples of pathogens that either alter the physiology or behavior of their insect hosts in ways that enhance their transmission. For example, *Xenopsylla cheopis* fleas carrying the plague bacilli suffer from Bacot's block, a plug of bacteria that prevents this insect from ingesting any blood (Bacot and Martin, 1914; Bacot, 1915). As a result, the infected flea regurgitates the bacilli into the host during its persistent but futile attempts to feed. A similar pattern has been found in *Leishmania donovani*-infected sandflies, which expel plugs of parasites during their multiple attempts to feed (Beach, 1985). Tsetse flies were discovered to probe the host significantly more often when infected with *Trypanosoma brucei*, and the female mosquitoes probed more frequently when infected with malaria parasites (Rossignol et al., 1984; Koella, 1997).

Rhodnius prolixus can be infected with another trypanosome, *Trypanosoma rangeli*. Though non-pathogenic to humans, *T. rangeli* is quite pathogenic to the insect host (Schaub, 1994). *Trypanosoma rangeli* is unique in that it can be transmitted both via saliva and feces (Hoare, 1972); the former mode is generally more efficient than the latter.

Anez and East (1984) also demonstrated that *T. rangeli*-infected bugs probed their hosts significantly more often than their uninfected counterparts.

Little research has been conducted to investigate whether *T. cruzi*-infection provides any cost or benefit to its insect host. Infected bugs appear to suffer few or no serious physiological effects (Schaub, 1988 and 1992), although high parasite burdens purportedly can kill the insect. *Trypanosoma cruzi*-infected insects may have delayed molting times or reduced reproductive capacity (Lima et al., 1992; Takano, unpublished results). D'Alessandro and Mandel (1969) also reported that *T. cruzi*-infected *R. prolixus* did not probe significantly more frequently than uninfected *R. prolixus*.

If the parasite manipulates its host in an attempt to enhance its own chances of transmission, there are several aspects of *R. prolixus* behavior that could be potential targets. Irritation of the host during feeding may increase transmission by causing the bugs to probe longer or to seek blood more frequently. In contrast, when permitted to feed uninterrupted, bugs may obtain larger blood meals and defecate more while still on the host, increasing the chances of pathogen transmission. Insects that feed quickly are generally selected for, since this behavior reduces the chance of detection by the host and of being crushed or eaten by the host.

If parasites caused bugs to defecate sooner during feeding, this would be an even more effective means to increase transmission. I tested this possibility with *T. cruzi*-infected *R. prolixus*. In my experiments, *R. prolixus* feeding and defecation behavior were observed both on an artificial membrane containing rabbit blood and on a live guinea pig host. Vector competence was determined with a defecation index (Zeledon et al., 1977). The original use of this index was to compare the vector competence of

various kissing bug species. The higher the index, the greater the theoretical vector potential of the triatomid bug.

$$\text{Defecation index} = \frac{\text{\% defecating within 10 min} \times \text{average no. of defecations in 10 min}}{100}$$

The original index used 10 min as the cut-off time, since the authors assumed that insects would remain in the proximity of the host for 5-10 min. Laboratory observations strongly suggest that, although many insects defecate before the completion of feeding, they leave the host within a few minutes. Thus, I altered the index to accommodate insect defecation behavior occurring from feeding initiation until 5 min post-feeding;

A final experiment compared the responses of infected and uninfected insects to a human hand in an olfactometer.

Materials and Methods

Insects

Rhodnius prolixus were maintained in the laboratory at 28° C, 80% humidity, and a 14L:10D photoregime. Third and 4th instar *R. prolixus* were starved for 3-5 wk after molting before they were artificially fed through a latex membrane containing an infective rabbit blood meal inoculated with *T. cruzi* (7.9×10^4 parasites/ml blood); virgin male and female adults were starved approximately 2 wk before infected (7.9×10^4 parasites/ml blood). Nymphal bugs were infected during their one and only blood meal at that

particular life stage. Typically, *R. prolixus* requires only one blood meal per life stage, except for the adults, which take multiple blood meals. Insects failing to feed or to molt were discarded from the study.

The two different feeding systems were employed for defecation observations:

1. Artificial membrane feeding trials utilized 5th instars (infected as 4th instars) that had been starved either 2-3 or 5-6 months.
2. Live host feeding trials used 4th (infected as 3rds), 5th instars (infected as 4ths) or adults (infected as newly emerged virgins). Fourth instars and adults were starved approximately 2-3 weeks after molting before experimental feedings began; fifth instars were starved for 2-5 weeks after molting. Though bugs may begin feeding 1 wk after molting, allowing at least 30 days to elapse from the last blood meal is usually beneficial in acquiring high percentages of feeding activity.

Animals

Six male guinea pigs (retired breeders, Hartley strain) were obtained from the Elm Hill Breeding Labs in Chelmsford, Massachusetts. The average weight of the guinea pigs was 1 kg.

Parasites

The Silvio strain of *T. cruzi* was used to initially infect *R. prolixus*. Methods for bug infection was described earlier in Chapter 2, and for parasite culture methods in Chapter 3.

Silvio *T. cruzi* trypomastigotes were obtained from Tufts University. Parasites were maintained at Tufts University 37° C and passed through Vero cell cultures daily. Cultures containing trypomastigotes were washed once or twice with 10% FCS-RPMI at 2400 rpm. Trypomastigotes were counted on a hemacytometer and an appropriate volume containing the desired number of parasites was added to the blood. Infected blood was packed on ice and transported back to the Medical Entomology Laboratory for immediate experimental use.

Experimental design for artificial membrane feeding system

Details of the artificial feeding apparatus were provided in Chapter 2. Feeding and defecation behavior of each infected and clean bug was observed and timed on the artificial membrane system. Factors recorded were: (1) total feeding time (min), (2) number of probing attempts, (3) time to first defecation relative to the completion of feeding (min) (feeding completion = t_0), (4) number of fecal drops discharged from feeding initiation to 5 min post-feeding, and (5) total blood meal weight (g). Insects were fed individually and, while experiments were in progress, the floor of the laboratory was artificially darkened to mimic the night-time when these nocturnal bugs normally feed in nature. Experimental feedings also coincided with the dark cycle of the insects' incubator. A 25-watt red light allowed observations to be conducted.

Dissection and microscopic examination to confirm infection took place approximately 4 wk after the final blood meal. Data for uninfected insects were discarded.

Three feeding regimes were studied (Table 8). Bugs were offered either one blood meal (feeding to repletion), two blood meals (one interrupted 2-min feeding, followed by a

second feeding to repletion the next day), or three blood meals (two interrupted 2-min feedings on two consecutive days, followed by a third feeding to repletion on day 3).

Experimental design for live host feeding

Feeding and defecation behavior of each infected and uninfected bugs when on a live host were observed and recorded. The same factors were compared as with the membrane experiments: (1) total feeding time, (2) number of probing attempts, (3) time to first defecation with regards to feeding completion (feeding completion = t_o), (4) number of defecations discharged from feeding initiation until 5 min post-feeding, and (5) total blood meal weight (g). Bugs were examined for infection approximately 4-8 wk after their final blood meal. Data for uninfected insects were discarded.

Two feeding regimes were studied (Table 8). Bugs were offered either one replete blood meal or two blood meals (one interrupted 2-min feeding, followed by a second feeding to repletion the next day).

Animal restrainer

The guinea pig restrainer was a rectangular tunnel structure (20.0 x 9.4 x 9.4 cm) manufactured from hardware cloth; the ends of the restrainer were hinged with Velcro straps and could be easily opened and closed. The hardware cloth tunnel was attached to a Plexiglas base (22 x 30 cm) using a glue gun. A hole (approximately 4.4 cm diameter) was cut in the Plexiglas base to permit bugs to feed through the hole on the guinea pig's belly. Four 3.75 cm-PVC tube adapters were glued to the Plexiglas base, elevating it enough to allow room for placement of the transparent cup containing the insect beneath

the hole. The restrainer was then situated on a plastic box to raise the restrainer to eye-level and was tilted to drain guinea pig urine onto the underlying newspapers.

Guinea pigs were encouraged to enter the restrainer by placing a piece of apple inside and the animals were permitted to finish eating the apple during the experiment. Once the guinea pig was placed into the hardware cloth restrainer, the doors were secured. The mesh-covered cup containing the bug was placed beneath the hole in the restrainer base, and the bug was allowed to feed. Timing commenced from the time feeding began and was recorded with a stopwatch. The initiation of feeding was determined as the moment when probing ceased for 10 sec. This was usually confirmed by the movement of the antenna to a perpendicular angle, which signified that the bug had successfully reached the blood source (Takano, unpublished results).

It was impossible to completely restrain the guinea pigs without causing them undue stress. Therefore, the guinea pigs would occasionally engage in grooming activity or shift their position slightly. If a guinea pig was unusually active, the bug was removed and the guinea pig unrestrained and the data were not included.

Olfactometer

Trypanosoma cruzi-infected and uninfected triatomids were tested individually within an olfactometer that consisted of a straight, enclosed Plexiglas tunnel, separated into two chambers; bugs were introduced into a refuge at one end of the track (37.5 x 3.1 x 6.3 cm). A human hand was placed in another chamber (22.5 x 10.6 x 6.3 cm), at the opposite end, separated from the track by a mesh screen to prevent bug contact.

The bug refuge consisted of a small plastic vial (3.1 cm diameter) open on one end and screened at the other (aluminum screening: 7 strands per cm); a small piece of folded paper towel was placed inside. Two strips of thin corking material were glued to two side of the vial, to prevent rolling. In general, the bugs remained placidly in their refuge until a stimulus was offered. A piece of brown paper was used to line the bug raceway, since triatomids appear to have some difficulty walking confidently on Plexiglas. All experiments were performed in a chemical hood with an estimated air flow of 3750 cm/min. For observational purposes, the hood light remained on. The insects did not display any reduced locomotory activity when the experiments were being performed during regular light hours.

Experimental design for olfactometer

Insects were timed in their approach to the human hand stimulus. Timing was stopped once the insect reached the mesh screen. The same host was used for all experiments. One infected bug was tested simultaneously with an uninfected bug, but within a separate olfactometer. Thus, both host hands were employed during the experiments. Insects were permitted 5 min to react to the stimulus before being excluded from the experiment.

Three different sets of starved 5th instar *R. prolixus* were used. Bugs were starved for either 1.5 months (n = 26-28), 4 months (n = 48-56), or 6 months (n = 13-16). Parasite infection of bugs starved 1.5 months and 6 months was confirmed by dissection and microscopic examination after the olfactometer tests were completed.

Results

Artificial-membrane feeding system (5th instars starved for 2-3 months)

Results from artificial membrane feeding are presented in Tables 9-11 for bugs ingesting 1 BM, 2 BM or 3 BM, respectively. Blood meal weights significantly differed ($p < 0.05$) between bugs that successfully molted and those that did not molt; therefore, the two groups were separated before statistical analysis.

Infected insects taking one or two blood meals and successfully molting to the adult stage defecated significantly more frequently than those infected insects that failed to molt (2.1 ± 0.3 vs. 1.5 ± 0.3 defecations (1 BM) and 1.5 ± 0.3 vs. 1.1 ± 0.3 defecations (2 BM)) ($p < 0.05$). Infected insects fed 2 blood meals and successfully molting had significantly fewer probing attempts than infected insects that failed to molt (3.7 ± 1.4 vs. 8.2 ± 2.9 probing attempts) ($p < 0.05$). A similar trend was found among uninfected insects fed 3 blood meals (1.92 ± 0.6 vs. 12.9 ± 3.3 probing attempts) ($p < 0.05$).

Among insects that successfully molted after two blood meals, infected ones defecated significantly more often prior to the completion of feeding than uninfected ones (1.0 ± 0.2 vs. 0.0 ± 0.0 defecations) ($p < 0.05$). Uninfected insects that ingested one blood meal and failed to molt had significantly fewer probing attempts than infected insects (8.8 ± 3.2 vs. 13.7 ± 1.5 probing attempts) ($p < 0.05$). Infected insects that successfully molted and after one or two blood meals, defecated significantly sooner than uninfected insects (-5.0 ± 2.1 min vs. 0.6 ± 0.2 min and -4.2 ± 2.5 min vs. -3.4 ± 2.8 min).

Defecation indices were higher for those bugs that successfully molted (Table 12). Indices for infected insects decreased as the number of blood meals increased.

Comparisons between the pooled indices of uninfected vs. infected insects did not yield any significant differences ($p > 0.05$).

Artificial-membrane feeding system (5th instars starved for 5-6 months)

Results with 5th instars are given in Tables 13-15 for bugs ingesting 1 BM, 2 BM, or 3 BM respectively. Because blood meal weights tended to differ significantly ($p < 0.05$) between bugs that successfully molted and those that did not molt, the two groups were statistically analyzed separately.

Uninfected insects that ingested one blood meal and successfully molting, passed significantly more fecal drops after feeding had been completed (1.2 ± 0.2 vs. 0.3 ± 0.1 defecations) ($p < 0.05$) and had significantly fewer interruptions than those insects that failed to molt (3.6 ± 1.2 vs. 9.6 ± 1.9 defecations) ($p < 0.05$) (Table 13).

Defecation indices for infected insects increased as the number of blood meals increased (Table 16). Indices for uninfected insects, remained relatively similar. Overall, higher indices were found among molting insects. Pooled defecation indices for uninfected insects did not significantly differ from pooled defecation indices of infected insects ($p > 0.05$).

Artificial-membrane feeding system summary

For insects that molted successfully, the total feeding time did not significantly differ between infected and uninfected insects, the number of blood meals, or the period of fasting ($p > 0.05$). However, among those insects that failed to molt, those fasted for 2-3

months fed for significantly shorter periods of time than insects fasted for 5-6 months (12.67 $\pm$ 0.76 min vs. 17.20 $\pm$ 1.18 min) ($p = 0.0001$).

The number of feeding interruptions differed significantly with the number of blood meals ingested by the bugs that failed to molt (9.65 $\pm$ 1.03 interruptions (1 BM) vs. 5.86 $\pm$ 1.20 interruptions (2 BM) ($p = 0.0018$). Insects that failed to molt had significantly more blood meal interruptions if fasted for 5-6 months than for 2-3 months (9.67 $\pm$ 1.16 interruptions vs. 6.47 $\pm$ 0.97 interruptions) ($p = 0.0362$).

Insects that fasted for 5-6 months and successfully molted defecated slightly earlier than those fasted for 2-3 months (-1.4 $\pm$ 0.5 min vs. 2.8 $\pm$ 1.0 min) ($p = 0.3431$). However, among non-molting insects, those fasted longer defecated significantly earlier (-3.3 min $\pm$ 0.9 vs. 5.9 $\pm$ 1.2 min) ($p > 0.0001$).

The total number of defecations produced from feeding initiation until 5 min post-feeding by infected and uninfected insects that successfully molted was statistically significant (1.15 $\pm$ 0.12 defecations vs. 0.15 $\pm$ 0.11 defecations) ($p = 0.057$). Unmolted insects starved 5-6 months defecated significantly less frequently than those starved 2-3 months (0.95 $\pm$ 0.10 defecations vs. 1.25 $\pm$ 0.15 defecations) ($p = 0.0431$).

Since the total blood meal weights did not significantly differ between infected and uninfected insects or by the number of blood meals, these groups were pooled. Insects that fasted 5-6 months had significantly smaller blood meal sizes than those fasted for 2-3 months (0.186 $\pm$ 0.006 g vs. 0.211 $\pm$ 0.005 g) ($p = 0.002$). A similar trend was found among insects that failed to molt (0.071 $\pm$ 0.005 g vs. 0.090 $\pm$ 0.009 g) ($p = 0.0096$).

Live host feeding

There were no significant differences between uninfected vs. infected insects ingesting either one or two blood meals, with regards to: (1) total feeding time, (2) number of probing attempts, (3) time elapsing before first defecation occurred, (4) number of fecal drops discharged within 5-minutes post-feeding, or (5) blood meal weight ($p > 0.05$) (Tables 17-20).

Data for male and female adult insects could not be pooled, due to significant differences arising from differences in their feeding behavior. Females, infected or uninfected, ingested a significantly larger blood meal when feeding to repletion than infected or uninfected males ($p < 0.05$). However, feeding times between the two sexes did not differ significantly ($p > 0.05$).

Data for adult male *R. prolixus* suggest that the number of blood meals ingested may have had a significant impact on the blood meal size ingested; males ingesting multiple meals ingested more blood than those ingesting only one meal (0.100 ± 0.004 g vs. 0.090 ± 0.002 g) ($p = 0.0627$).

The number of blood meals ingested also had a significant impact on the defecation time of 5th instars; those ingesting two blood meals defecated significantly sooner than those ingesting one blood meal (-10.1 ± 1.2 min vs. -6.86 ± 1.11 min) ($p = 0.0504$). Infected fifth instars also defecated less frequently than their uninfected counterparts (4.6 ± 0.5 defecations vs. 3.4 ± 0.5 defecations) ($p = 0.0739$).

Defecation indices

Taking two blood meals appeared to increase the vector competence of infected, but not uninfected bugs (Table 21). The indices for uninfected insects increased only slightly, but the increase was more dramatic for infected bugs. However, among insects ingesting only one blood meal, the defecation indices for infected bugs were consistently lower than those of uninfected insects.

Only the defecation indices of fifth instars can be compared over all fasting periods. Insects starved 2-5 weeks had significantly higher defecation indices than those insects starved 2-3 or 5-6 months (4.0 ± 0.4 vs. 1.2 ± 0.1 or 1.3 ± 0.1) ($p < 0.05$).

Fifth instars had the highest defecation indices of all life stages studied (4.0 ± 0.4), which were significantly higher than defecation indices for 4th instars (2.2 ± 0.2) or adult males (0.6 ± 0.1) ($p < 0.05$), but not adult females (2.7 ± 0.5). However, the defecation indices for adult females were significantly greater than those of adult males ($p < 0.05$).

Olfactometer

The results of olfactometer trials are in Table 22. One hundred percent of all bugs dissected in the groups starved for 1.5 months or 6 months were infected. It was assumed, therefore, that all insects starved for 4-6 months were also infected, even though these were not dissected. There was a higher response rate from infected bugs (starved 4 months) vs. uninfected bugs (82% vs. 71%) but a higher response rate from uninfected bugs (starved 6 months) vs. infected bugs (84% vs. 68%). Infected bugs starved for 4-6 months were faster than uninfected bugs, but not after starvation for 1.5 months. There

were no significant differences of response levels between infected and uninfected insects starved for various periods of time.

Table 8. Feeding regimes for artificial-membrane feeding system and live host feeding system.

ARTIFICIAL-MEMBRANE SYSTEM

Feeding Schedule	Day 1	Day 2	Day 3
1	feed to repletion		
2	feed 2-min	feed to repletion	
3	feed 2-min	feed 2-min	feed to repletion

LIVE HOST SYSTEM

Feeding Schedule	Day 1	Day 2
1	feed to repletion	
2	feed 2-min	feed to repletion

Table 9. Defecation and feeding parameters of 5th instar *Rhodnius prolixus* ingesting one blood meal (1 BM) after fasting 2-3 months.

Molted	Uninfected Bugs (n = 10)	Infected Bugs (n = 7)
Mean total feeding time (min)	18.02 ± 1.74	19.66 ± 2.60
No. probing attempts	3.10 ± 0.93	6.71 ± 3.01
Time to first defecation (min)*	0.60 ± 0.18[b]	-5.00 ± 2.07[b]
No. fecal drops 5 min post-feeding	0.90 ± 0.17	2.14 ± 0.34[a]
Total BM weight (g)	0.22 ± 0.01	0.22 ± 0.01

Unmolted	Uninfected Bugs (n = 9)	Infected Bugs (n = 13)
Mean total feeding time (min)	17.03 ± 3.66	18.50 ± 2.95
No. probing attempts	8.78 ± 3.24	13.77 ± 1.52
Time to first defecation (min)*	-8.53 ± 4.11	-5.91 ± 1.97
No. fecal drops 5 min post-feeding	1.33 ± 0.53	1.15 ± 0.30[a]
Total BM weight (g)	0.10 ± 0.02	0.05 ± 0.01

* - = minutes before feeding completion; + = minutes after feeding completion
[a,b] = $p < 0.05$

Table 10. Defecation and feeding parameters of 5th instar *Rhodnius prolixus* ingesting two blood meals (2 BM) after fasting 2-3 months.

Molted	Uninfected Bugs (n = 13)	Infected Bugs (n = 10)
Mean total feeding time (min)	18.30 ± 2.21	17.12 ± 2.41
No. probing attempts	3.77 ± 1.22	3.70 ± 1.37[b]
Time to first defecation (min)*	-3.35 ± 2.80[c]	-4.22 ± 2.47[c]
No. fecal drops 5 min post-feeding	1.46 ± 0.31	1.50 ± 0.33
Total BM weight (g)	0.20 ± 0.01	0.19 ± 0.01[a]

Unmolted	Uninfected Bugs (n = 7)	Infected Bugs (n = 13)
Mean total feeding time (min)	18.17 ± 3.13	16.70 ± 2.69
No. probing attempts	6.89 ± 2.97	8.15 ± 2.91[b]
Time to first defecation (min)*	-7.29 ± 3.12	-4.65 ± 2.53
No. fecal drops 5 min post-feeding	1.33 ± 0.50	1.08 ± 0.26
Total BM weight (g)	0.11 ± 0.02	0.09 ± 0.02[a]

* - = minutes before feeding completion; + = minutes after feeding completion
[a,b,c] = $p < 0.05$

Table 11. Defecation and feeding parameters of 5th instar *Rhodnius prolixus* ingesting three blood meals (3 BM) after fasting 2-3 months.

Molted	Uninfected Bugs (n = 13)	Infected Bugs (n = 10)
Mean total feeding time (min)	16.81 ± 1.16	18.30 ± 2.45
No. probing attempts	1.92 ± 0.59[a]	1.50 ± 0.46
Time to first defecation (min)*	-0.32 ± 0.44	-2.44 ± 2.08
No. fecal drops 5 min post-feeding	0.85 ± 0.09	1.1 ± 0.15
Total BM weight (g)	0.21 ± 0.01	0.22 ± 0.02

Unmolted	Uninfected Bugs (n = 7)	Infected Bugs (n = 6)
Mean total feeding time (min)	19.79 ± 2.08	15.03 ± 2.03
No. probing attempts	12.86 ± 3.27[b]	5.83 ± 2.59
Time to first defecation (min)*	-7.10 ± 2.46	-2.86 ± 1.71
No. fecal drops 5 min post-feeding	1.43 ± 0.28	1.33 ± 0.20
Total BM weight (g)	0.11 ± 0.02	0.11 ± 0.02

* - = minutes before feeding completion; + = minutes after feeding completion
[a,b] = $p < 0.05$

Table 12. Defecation indices of 5th instar *Rhodnius prolixus* starved 2-3 months.

UNINFECTED INSECTS

#BM's	Molted?	Mean No. Defecations Until 5 min Post-Feeding	% Defecating 5 min Post-Feeding	INDEX
1	No	2.00	66.67	1.33
	Yes	1.13	80.00	0.90
2	No	1.71	77.78	1.33
	Yes	1.46	100.00	1.46
3	No	1.67	85.71	1.43
	Yes	1.10	76.92	0.85

INFECTED INSECTS

#BM's	Molted?	Mean No. Defecations Until 5 min Post-Feeding	% Defecating 5 min Post-Feeding	INDEX
1	No	1.40	76.92	1.08
	Yes	2.14	100.00	2.14
2	No	1.56	69.23	1.08
	Yes	1.67	90.00	1.50
3	No	1.33	100.00	1.33
	Yes	1.22	90.00	1.10

Table 13. Defecation and feeding parameters of 5th instar *Rhodnius prolixus* ingesting one blood meal (1 BM) after fasting 5-6 months.

Molted	Uninfected Bugs (n = 5)	Infected Bugs (n = 8)
Mean total feeding time (min)	17.79 ± 2.89	15.10 ± 1.50
No. probing attempts	3.60 ± 1.22[a]	2.63 ± 1.22
Time of first defecation (min) *	-3.08 ± 1.26	-1.88 ± 1.31
No. fecal drops 5 min post-feeding	1.8 ± 0.58	1.25 ± 0.16
Total BM weight (g)	0.19 ± 0.01	0.18 ± 0.01

Unmolted	Uninfected Bugs (n = 16)	Infected Bugs (n = 13)
Mean total feeding time (min)	15.74 ± 1.38	12.43 ± 1.14
No. probing attempts	9.62 ± 1.90[b]	6.15 ± 1.43
Time to first defecation (min)*	-4.75 ± 1.32	-2.54 + 1.16
No. fecal drops 5 min post-feeding	0.88 ± 0.22	0.85 ± 0.22
Total BM weight (g)	0.08 ± 0.02	0.08 ± 0.02

* - = minutes before feeding completion; + = minutes after feeding completion
[a,b] = $p < 0.05$

Table 14. Defecation and feeding parameters of 5th instar *Rhodnius prolixus* ingesting two blood meals (2 BM) after fasting 5-6 months.

Molted	Uninfected Bugs (n = 1)	Infected Bugs (n = 11)
Mean total feeding time (min)	14.37	15.31 ± 1.53
No. probing attempts	4.00	1.63 ± 0.64
Time of first defecation (min)*	-0.02	-0.87 ± 0.85
No. fecal drops 5 min post-feeding	1.00	1.36 ± 0.20
Total BM weight (g)	0.18	0.19 ± 0.01

Unmolted	Uninfected Bugs (n = 17)	Infected Bugs (n = 11)
Mean total feeding time (min)	10.85 ± 1.74	9.44 ± 1.87
No. probing attempts	5.00 ± 2.05	3.64 ± 1.42
Time to first defecation (min)*	-3.55 ± 1.82	-2.41 ± 1.34
No. fecal drops 5 min post-feeding	1.06 ± 0.23	0.91 ± 0.25
Total BM weight (g)	0.08 ± 0.01	0.06 ± 0.01

* - = minutes before feeding completion; + = minutes after feeding completion

Table 15. Defecation and feeding parameters of 5th instar *Rhodnius prolixus* ingesting three blood meals (3 BM) after fasting 5-6 months.

Molted	Uninfected Bugs (n = 4)	Infected Bugs (n = 8)
Mean total feeding time (min)	14.83 ± 1.87	16.11 ± 2.90
No. probing attempts	4.25 ± 2.32	1.75 ± 0.94
Time to first defecation (min)*	-1.58 ± 1.75	-1.47 ± 1.29
No. fecal drops 5 min post-feeding	2.00 ± 0.71	2.00 ± 0.63
Total BM weight (g)	0.15 ± 0.01	0.20 ± 0.02

Unmolted	Uninfected Bugs (n = 10)	Infected Bugs (n = 11)
Mean total feeding time (min)	11.74 ± 1.16	12.92 ± 1.25
No. probing attempts	5.00 ± 1.59	8.73 ± 4.64
Time to first defecation (min)*	-0.68 ± 0.10	-4.76 ± 2.97
No. fecal drops 5 min post-feeding	0.90 ± 0.20	1.09 ± 0.28
Total BM weight (g)	0.06 ± 0.02	0.09 ± 0.02

* - = minutes before feeding completion; + = minutes after feeding completion

Table 16. Defecation indices of 5th instar *Rhodnius prolixus* starved 5-6 months.

UNINFECTED INSECTS

#BM's	Molted?	Mean No. Defecations Until 5 min Post-Feeding	% Defecating 5 min Post-Feeding	INDEX
1	No	1.4	62.50	0.88
	Yes	1.4	100.00	1.40
2	No	1.5	70.60	1.06
	Yes	1.0	100.00	1.00
3	No	1.3	70.00	0.90
	Yes	1.5	100.00	1.50

INFECTED INSECTS

#BM's	Molted?	Mean No. Defecations Until 5 min Post-Feeding	% Defecating 5 min Post-Feeding	INDEX
1	No	1.22	69.20	0.85
	Yes	1.25	100.00	1.25
2	No	1.43	63.60	0.91
	Yes	1.36	100.00	1.36
3	No	1.50	72.70	1.09
	Yes	1.63	100.00	1.63

Table 17. Defecation and feeding parameters of adult female *Rhodnius prolixus* ingesting one or two blood meals, fasted for 2-3 wk.

1 BM	Uninfected Bugs (n = 15)	Infected Bugs (n = 11)
Mean total feeding time (min)	15.63 ± 1.58	16.46 ± 2.55
No. probing attempts	3.00 ± 0.68	2.62 ± 0.52
First defecation (min)*	-4.58 ± 1.41	-4.13 ± 1.77
No. fecal drops	2.40 ± 0.49	2.00 ± 0.37
Total blood meal (g)	0.15 ± 0.01	0.15 ± 0.01

2 BM	Uninfected Bugs (n = 12)	Infected Bugs (n = 11)
Mean total feeding time (min)	17.88 ± 3.55	19.91 ± 5.27
No. probing attempts	4.92 ± 1.73	2.90 ± 1.41
First defecation (min)*	-7.86 ± 3.41	-10.30 ± 3.89
No. fecal drops	2.58 ± 0.50	4.18 ± 1.38
Total blood meal (g)	0.16 ± 0.01	0.16 ± 0.01

* - = minutes before feeding completion; + = minutes after feeding completion

Table 18. Defecation and feeding parameters of adult male *Rhodnius prolixus* ingesting one or two blood meals, fasted for 2-3 wk.

1 BM	Uninfected Bugs (n = 16)	Infected Bugs (n = 11)
Total feeding time (min)	11.31 ± 1.53	11.79 ± 1.19
No. probing attempts	1.94 ± 0.64	0.92 ± 0.45
First defecation (min)*	0.02 ± 0.39	3.78 ± 2.19
No. fecal drops	0.75 ± 0.11	0.62 ± 0.27
Total blood meal (g)	0.09 ± 0.00	0.09 ± 0.00

2 BM	Uninfected Bugs (n = 11)	Infected Bugs (n = 12)
Total feeding time (min)	10.87 ± 1.33	12.10 ± 1.64
No. probing attempts	1.64 ± 0.56	2.33 ± 0.56
First defecation (min)*	-0.88 ± 1.42	1.33 ± 1.37
No. fecal drops	0.91 ± 0.28	1.00 ± 0.25
Total blood meal (g)	0.10 ± 0.00	0.10 ± 0.00

* - = minutes before feeding completion; + = minutes after feeding completion

Table 19. Defecation and feeding parameters of 4th instar *Rhodnius prolixus* ingesting one or two blood meals, fasted for 2-3 wk.

1 BM	Uninfected Bugs (n = 33)	Infected Bugs (n = 30)
Mean total feeding time (min)	18.98 ± 1.13	18.06 ± 3.10
No. probing attempts	4.06 ± 1.04	3.50 ± 1.22
First defecation (min)*	-3.54 ± 0.92	-3.97 ± 2.62
No. fecal drops	2.27 ± 0.20	1.50 ± 0.27
Total blood meal (g)	0.09 ± 0.00	0.08 ± 0.00

2 BM	Uninfected Bugs (n = 28)	Infected Bugs (n = 30)
Mean total feeding time (min)	20.09 ± 1.61	20.31 ± 1.57
No. probing attempts	5.93 ± 1.38	2.93 ± 0.58
First defecation (min)*	-5.36 ± 1.17	-5.37 ± 1.50
No. fecal drops	2.61 ± 0.34	2.60 ± 0.46
Total blood meal (g)	0.09 ± 0.00	0.09 ± 0.00

* - = minutes before feeding completion; + = minutes after feeding completion

Table 20. Defecation and feeding parameters of 5th instar *Rhodnius prolixus* ingesting one or two blood meals, fasted for 2-3 wk.

1 BM	Uninfected Bugs (n = 32)	Infected Bugs (n = 34)
Total feeding time (min)	27.89 ± 1.70	23.40 ± 1.78
No. probing attempts	6.09 + 1.22	4.59 + 0.85
First defecation (min)*	-8.51 ± 1.65	-5.28 ± 1.45
No. fecal drops	4.28 ± 0.71	2.97 ± 0.49
Total blood meal (g)	0.28 ± 0.01	0.26 ± 0.01

2 BM	Uninfected Bugs (n = 31)	Infected Bugs (n = 33)
Total feeding time (min)	27.40 ± 1.61	26.50 ± 1.77
No. probing attempts	7.39 ± 1.72	5.70 ± 0.88
First defecation (min)*	-10.05 ± 1.58	-10.15 ± 1.82
No. fecal drops	4.97 ± 0.69	3.88 ± 0.76
Total blood meal (g)	0.29 ± 0.01	0.28 ± 0.01

* - = minutes before feeding completion; + = minutes after feeding completion

Table 21. Defecation indices for live host feeding experiment.

Description (#BM's)	Mean # Defecations Until 5 min Post-Feeding	% Defecating 5 min Post-Feeding	INDEX
4th instars[1]			
Uninfected (1 BM)	2.27 ± 0.20	100.00	2.27
Infected (1 BM)	1.50 ± 0.27	100.00	1.50
Uninfected (2 BM)	2.61 ± 0.34	96.43	2.52
Infected (2 BM)	2.60 ± 0.46	90.00	2.34
5th instars[2]			
Uninfected (1 BM)	4.28 ± 0.71	100.00	4.28
Infected (1 BM)	2.97 ± 0.49	96.97	2.88
Uninfected (2 BM)	4.97 ± 0.70	100.00	4.97
Infected (2 BM)	3.88 ± 0.76	100.00	3.88
Adult females[1,2]			
Uninfected (1 BM)	2.40 ± 0.49	92.86	2.24
Infected (1 BM)	2.00 ± 0.35	92.31	1.82
Uninfected (2 BM)	2.58 ± 0.50	100.00	2.60
Infected (2 BM)	3.83 ± 1.26	100.00	4.20
Adult males[1]			
Uninfected (1 BM)	0.75 + 0.11	75.00	0.60
Infected (1 BM)	0.62 ± 0.27	38.46	0.32
Uninfected (2 BM)	0.91 ± 0.29	63.63	0.57
Infected (2 BM)	1.00 ± 0.25	75.00	0.75

[1,2] = significantly different ($p < 0.05$)

Table 22. Response rates of *Trypanosoma cruzi*-infected vs. uninfected *Rhodnius prolixus* to a human stimulus during olfactometer trials.

Bug Status	n =	Fasting Period	Time to Screen (sec)	% Response
Infected	13	6 months	79.08 ± 18.44	68%
Uninfected	16	6 months	90.63 ± 19.58	84%
Infected	56	4 months	81.41 ± 8.14	82%
Uninfected	48	4 months	101.41 ± 9.91	71%
Infected	28	1.5 months	111.54 ± 14.79	72%
Uninfected	26	1.5 months	98.81 ± 11.84	67%

Discussion

It is not possible to directly compare results from the artificial feeding system and the live host. Artificially fed insects were fed citrated rabbit blood and were starved longer for extended periods of time. Also, host movement during live host feedings was impossible to eliminate and most likely extended feeding times. However, this is likely to occur in nature as well.

Artificially fed insects were less likely to molt successfully into the next instar. This was probably related to the extended starvation period, since a high proportion of insects starved 3-5 weeks molted to the next instar when fed artificially. Insects were separated into molted and unmolted groups, since the physical difference after feeding was so obvious.

Insects fed on a live host produced higher BM/bug weight ratios. The few insects that failed to molt did not have noticeably lower BM/bug weight ratios. There appears to be no clear threshold of BM/bug weight ratio after which molting success is guaranteed. Distension of the abdomen is insufficient by itself to trigger molting. Nutrition difference between rabbit and guinea pig blood is a possible compounding factor since other laboratories have reported that *R. prolixus* thrives best on rabbit or human blood. Nonetheless, guinea pigs are a natural host of *R. prolixus* in South America. The extended fasting period of the artificially-fed insects was most likely responsible for many of differences found between artificially-fed vs. naturally-fed insects.

Mean total feeding time

Bugs starved for longer periods (5-6 months) and failing to molt took significantly less time to feed than insects fasted for only 2-3 months. Though it is difficult to determine trends between the two systems of feeding, my data suggest that the parameter of total feeding time tends to decrease over extended periods of fasting. In terms of vector competence, this means that the more starved a bug is, the less contact it will have with a host when it finally feeds.

Number of probing attempts

Bugs unable to successfully molt to the next stage probed more frequently; the longer the starvation period, the more the number of probing attempts increased. Repeated probing increases the chance of host irritation and probably increases the chance that defecation will occur while the bug is still on the host.

Time to first defecation

Longer fasting periods caused bugs to defecate earlier, with regards to the completion of feeding. Also, bugs that ingested two meals defecated significantly earlier than those that ingested only one. Thus, it appears that both fasting and multiple feeding can influence the timing of bug defecation and thereby, affect its vector potential.

Number of fecal drops discharged from feeding initiation to 5 min post-feeding

Infected 5th instars fasted for extended periods of time (2-3 or 5-6 months) defecated significantly more often than their uninfected counterparts. However, this trend

was reversed among 5th instars fed on a live host; infected insects defecated significantly less frequently than uninfected bugs. These data suggest that *T. cruzi* transmission may be enhanced if the bug has been starved for at least two months.

Total blood meal weight

A longer fasting period significantly reduced the total volume of blood ingested, regardless of whether or not the bug successfully molted. The fact that bugs fasted for longer periods feed for significantly less time than less-starved insects may be due to the reduced volume of blood ingested. My data suggest that the more frequently a bug is permitted to feed, the greater total volume of blood it will ingest. Therefore, both fasting period and multiple feeding affect the total amount of blood ingested. Both fasting period and multiple feeding can therefore influence bug vector potential.

Defecation indices

Only the data for 5th instars can be compared between the varying fasting levels. Bugs fasted for only 2-5 weeks had significantly higher defecation indices than insects starved for extended periods of time (2-3 or 5-6 months). If the biting frequency is lower during the cool winter season, one might expect an increase of Chagas' transmission once biting activity has become more frequent and the bugs have a higher defecation index. According to the defecation indices, 5th instars were by far the best vectors, followed by adult females, fourth instars and finally, adult males.

In evolutionary terms, selective pressure for *T. cruzi* to manipulate *R. prolixus* defecation behavior may not be great, since the close relationship of *R. prolixus* and the

dwellings of humans is probably quite recent. In nature, *R. prolixus* is an opportunistic feeder of nesting species. Infection of mammals, that act as reservoirs of *T. cruzi*, is more to occur through the ingestion of infected insects than through defecated feces. Even within the human dwelling, *R. prolixus* has the opportunity to feed on animals housed within or near the habitat, such as dogs, cats, chickens, and guinea pigs. Humans are one of several available hosts.

Levels of response to human stimulus

My data suggest that the more starved bugs are, the faster they respond to a host stimulus. It is difficult to conclude that infected bugs might be more sensitive to host odors than uninfected bugs and therefore react more quickly to them, since the percentage of responding infected bugs drops below that of uninfected bugs at 6 months of starvation. Though starvation can increase the vector competence of *R. prolixus* by affecting its defecation behavior, there appears to be a threshold after which the activity levels are adversely affected by starvation. Vector competence is a compilation of many environmental, physical and behavioral factors; one factor, such as starvation, may increase one aspect of vector competence but decrease another.

Impact of the parasite on *Rhodnius prolixus*

The gut of reduviids contains many enzymes and proteins. The presence of *T. cruzi* parasites could alter the environment of the bug gut in two ways: (1) parasite presence could stimulate the production of gut proteins by the bug system or (2) the parasite itself may produce and release substances that affect the bug gut. The density of

the parasite may also be important, since it has been demonstrated that *T. cruzi* affects the production of amino acids in reduviids (Schaub, 1992). The parasite must also compete with the bug for metabolites; this interaction could be compounded by the physiological state or life stage of the bugs, since different energy reserves are used for different purposes.

Implications of fasting period and vector competence

Although reports suggest that high proportions of *R. prolixus* can spontaneously abort their infection when fasted for at least two months, my bug colony did not follow that trend. Almost 100% of all insects starved 5-6 months maintained their infection. The ability of *T. cruzi* to withstand extended starvation stress within the bug, raises new questions about the vector competence and reservoir role of these bugs.

My data suggest that extended starvation can actually increase vector competence parameters such as defecation time and host irritation. The biting frequency of bugs varies by season (Catala et al., 1991); biting activity during the winter months can be much reduced. Also, bug fasting may result as a consequence of high bug densities; large numbers of bugs attempting to feed will ultimately drive host irritation to levels that permit bug feeding only under extremely dangerous conditions. The more resistant a bug is to starvation, the greater its chances for survival. However, extended starvation may also decrease an infected bug's ability to respond to host stimuli; or, perhaps the parasites drain precious metabolites, weakening the bug.

Under conditions of high population density, kissing bugs take smaller blood meals, probably as a direct result of increased host irritation (Kirk and Schofield, 1987),

and are unable to reach the next instar immediately. Failure to reach the next instar holds important implications for vector competence. By taking smaller blood meals, bugs are forced to feed more frequently, thereby increasing their potential to defecate on the host and transmit *T. cruzi* pathogens. Others have studied multiple blood feeding directly after the interruption of feeding. I took a different approach and investigated the impact of interruption on the next available blood meal. In general, bugs that feeding more frequently are more likely to defecate on or near the host than bugs that feed once to repletion. In nature, bugs are less likely to obtain adequate amounts of blood at one time, unless insect densities are sufficiently low to maintain moderate or lower levels of host irritation.

My data suggest that infection by *T. cruzi* somehow reduces the defecation activity of *R. prolixus* fed only one blood meal; defecation indices for infected bugs fed one blood meal were consistently lower than those of uninfected bugs. As the number of blood meals increases (representing prior interrupted blood meals and host irritation), the vector competence of *T. cruzi*-infected bugs quickly increases; in contrast, the vector competence of uninfected bugs remains constant.

CHAPTER VI

MOVEMENT AND ATTRACTION OF LABORATORY *RHODNIUS PROLIXUS* IN A SIMULATED FIELD ENVIRONMENT

Introduction

Studies on the dispersal behavior of *Rhodnius prolixus* have not been performed since the 1960's, when a few studies on the movement and preferential resting sites of *R. prolixus* found in houses of Argentina were performed. Gomez-Nunez (1965) found that most *R. prolixus* were found in the bedroom, close to the host. In mark-release-recapture experiment using ^{60}Co-tagged bugs, bugs released inside did not migrate outside though bugs released outside were found inside (Gomez-Nunez, 1969).

More detailed studies of dispersal and resting site preferences have been performed on *Triatoma infestans*, which is considered to be the most important vector of Chagas' disease due to its wide geographic distribution and close relationship with man. *Rhodnius prolixus* and *T. infestans* are not found in the same geographic regions, though both are domiciliated, so direct comparisons of interspecific competition cannot be observed. The same sampling devices and collection techniques have been used for both of these species, but the behavior of *T. infestans* has been more thoroughly studied. Though both species invade the peridomestic environment, *R. prolixus* is infamous for its aggressiveness and tenacity. Also, *R. prolixus* is known to prefer huts with thatched roofs and glues its eggs to the substrate while *T. infestans* does not display such strong preferences and simply drops its eggs near or at resting sites (Schofield, 1977). However, the type of roofing

material and indoor wall construction was found to be significantly associated with *T. infestans* density (Gurtler et al., 1992). Recent studies have suggested that *T. infestans* actively leave their refuges to purposefully drop their feces outside of the refuge, perhaps signaling a safe haven to other bugs (Lazzari et al., 1993) or concentrating the location of feces containing the necessary gut symbionts. Similar trends have not been studied with *R. prolixus*.

Field experiments to study bug behavior are difficult for a variety of reasons. The only ways to quantitate bug densities are to use sampling devices, man-hours of capture, or house demolition. Sampling boxes have limitations at lower bug densities, since they consist of satisfactory, but not overly attractive, refuges. Various sampling devices include Gomez-Nunez boxes (31 cm x 25 cm x 5 cm cardboard boxes with twenty 2-cm diameter circles cut out in the back) and other boxes of similar construction (Gomez-Nunez, 1965; Wisnivesky-Colli et al., 1987), which contain several pieces of crumpled paper towels or cardboard, and white sheets of typing paper tacked onto the wall (Garcia-Zapata, 1985). The use of man-hours to collect insects is often subjective and heavily influenced by the experience and energy of the collector. There are potential ethical dilemmas created by mark-release-recapture experiments, since additional vectors may be added to the existing household population. House demolition is an effective means of quantitating bug density but is expensive and impractical. Efficient bug sampling must be done in a simple yet reliable way that can easily be performed by the average homeowner for vigilance purposes.

Over the last several years, there has been much speculation about possible attractants for kissing bugs. Most studies have focused on *T. infestans*; and out of these

studies, two major schools of thought have emerged. One cites evidence that ammonia gas attracts *T. infestans* (Taneja and Guerin, 1997), which has implications for the importance of feces. The other argues that carbon dioxide produced by Baker's yeast has attractant qualities, which would exploit the bug's host-seeking behavior (Guerenstein et al., 1995). Schofield, during the 1970's, performed some experiments demonstrating that feces 3 hours to 12 days old are attractive to nymphs (Schofield and Patterson, 1977). However, the feasibility of using such attractants in the field has not been quantitated.

My objectives were to: (1) develop a quick monitoring device to detect bug infestation within the simulated field system, (2) determine potential bug attractants that could be incorporated into the bug monitoring device, and (3) test the use of the simulated field system to evaluate the use of potential bug attractants. A simulated field environment provides the opportunity to test the attraction of kissing bugs to various experimental components in the laboratory, by representing a controlled and duplicable situation slightly more realistic than an olfactometer. Therefore, I attempted to mimic the field environment, simulating the mud substrate commonly utilized in houses in Latin America.

Materials and Methods

Insects

Rhodnius prolixus were maintained in the laboratory at 28° C and 70-80% relative humidity at a 14L:10D photoregime. Kissing bugs (3rd, 4th and 5th instars) were starved at least 4-8 wk before use in these experiments.

Simulated field environment box

Artificial environments were created by gluing and caulking together 5 pieces of 61 x 61 x 61 cm white Styrofoam. Once the cubic structure was developed, its interior was coated with Sculptamold (American Art Clay Company), a synthetic substance designed to jointly embody the light-weight properties of clay and durability of plaster. Mixing ratios of material were 2:1 (Sculptamold:water). Between experiments, the cube was scraped with a metal spatula to remove fecal traces and washed with water. If necessary, touch-ups with Sculptamold were performed and permitted to dry.

Small artificial refuges were provided within the cubic structure to monitor levels of bug recruitment. Refuges were held in place within the system by clear pushpins or if necessary, glue. The final side of the cubic structure was covered by three strips of clear plastic wrap, secured by thumbtacks. The middle strip was easily movable, allowing for the introduction of kissing bugs, attractants and the removal of refuges and bugs at the end of the experiment.

Refuges

Different types of refuges were utilized in the following formats:

Format A: Small Gomez-Nunez (GN) boxes (7.5 x 7.5 x 2.5 cm high with nine 1.25 cm diameter holes on one side) were compared against single fiberpots (4.4 x 4.4 (upper base) x 3.1 x 3.1 (lower base) x 5 cm (height)). Each fiberpot contained two contiguous hole-punches (0.6 cm diameter) near the base that provided 4-way entry for the insects. In experiments in which fasted insects were offered the choice of one small GN box or one single fiberpot, the small GN box was slightly elevated to permit bug entry

by gluing two wooden applicator sticks (ca. 7.5 cm long) to two sides of the refuge bottom.

Format B: Large GN boxes (18.1 x 6.9 x 3.1 cm) with ten holes on one side) were compared against a series of four fiberpots glued together. Each fiberpot contained four entrances as described above. GN boxes were of different texture and color: (1) white typing paper, (2) cream construction paper, or (3) black construction paper.

Format C: Larger fiberpot refuges were formed by gluing three single fiberpots together, each with four entrances. Some refuges were supplemented with a potential attractant.

Attractants

One gram of yeast was mixed with one gram of sugar plus 10 ml distilled water. Four hundred µl of this were added to a modified 1.7 ml microcentrifuge tube (the cap was glued to the bottom of the tube and the top of the tube was cut at the 1.0 ml mark). Control tubes had 400 µl of distilled water.

As ammonia hydroxide evaporates, ammonia gas is released. Since NH_4OH consists of 28 - 29% pure NH_3, 2.7×10^{-7} moles were required to make 10 ml of a 24 µM NH_3 solution (24 µl of a 1,000-fold dilution of NH_4OH) while 10 ml of a 240 µM solution required 2.4×10^{-6} moles (240 µl of a 1,000-fold dilution of NH_4OH). All NH_4OH solutions were stored in the refrigerator at 6° C until use. Four hundred µl of each solution was used as an attractant, while 400 µl of distilled water served as the control.

Colony feces were tested in the following ways, to examine whether *R. prolixus* feces contained any attractive chemical cues: (1) passive air test (small plastic

box), (2) Y-olfactometer, or (3) dual-port petri dish. A variable number of bugs (1, 5, or 10) was placed in a small clear plastic box (12 x 7 x 5 cm) with 4 lateral screens (2 cm diameter) containing clean and feces-coated filter paper was also used. Each box contained both clean and feces-coated folded quarter-circles of 10.75 cm diameter filter paper. Variable numbers of bugs were used to examine whether bug density affects bug choice. Bugs were monitored once a week for 4 wk, during which time the number of bugs resting on the pieces of filter paper were observed and recorded. Four replicates of each group were run at the same time. Bugs not resting on either filter paper were not counted. Colony feces used for one year by fourth instars but lacking fresh blood meal defecations within the last month, were tested in a dual-choice petri dish overnight and in a Y-olfactometer for 1 hr. Fresh feces also were tested for bug attractiveness in the Y-olfactometer aged for 3, 6, 12, and 21 hr; insects were permitted 1 hr to make a choice.

Artificial host

Artificial hosts consisted of a Repti Therm heating mat (Zoo Med Laboratories, Inc.) on which a blood meal sealed within a Parafilm envelop was laid. After approximately 10 min, the heating mat attained a temperature comparable to a live human and was introduced into the system for 5 min: a cup containing bugs was inverted on the envelope. After 5 min had elapsed, the cup was removed and bugs were permitted to continue feeding if they chose. Once all bugs had stopped feeding, the blood-filled envelope was removed.

Olfactometers

Two different types of olfactometers were utilized in these studies: (1) a Y-shaped olfactometer, in which cues were placed at each end of the Y, and (2) a petri dish olfactometer with dual-ports.

The Y-shaped olfactometer was constructed of 0.3 cm-thickness Plexiglas and consisted of four major parts: (1) a base in which chilled bugs were placed (10 x 16.6 x 8 cm); (2) a middle zone to which the two arms were glued ($base_1$ = 10 cm; $base_2$ = 20 cm; height = 8 cm and width = 10 cm); (3) two arms, each measuring 25 x 8.4 x 8 cm; and (4) a screened-off box in which attractants could be detected by insects but not contacted (7.5 x 10 x 8 cm).

The dual-port petri dish olfactometers, modified from Prokopy et al. (1995), consisted of two 1 oz clear plastic cups, a 8.9 cm diameter petri dish and two strips of white construction paper 1.25 x 5 cm. Two 1.6 cm holes were bored into the lid of the petri dish with a heated cork borer; construction paper was rolled up and placed into each hole and a plastic cup was inverted over each paper/hole. Attractants were introduced beneath the plastic cup, but outside of the paper; bugs were defined as having made a "choice" if located on either piece of paper leading up to an experimental substance or the control. Experimental substances in liquid attractants (400 µl volume) were placed in modified 1.7 ml microcentrifuge tubes; colony feces were tested on small, folded pieces of filter paper.

Overnight dispersal of nymphs: comparison of two refuges

Experiments were run overnight with either a combination of: (1) 10 3rd, 10 4th, and 10 5th instars or (2) 25 3rd and 10 5th instars for each system, supplemented with refuges according to Format A. Seventeen refuges of one type only were placed within various strata of the system: the lower and upper wall (7 and 7 refuges, respectively), in addition to the ceiling (3 refuges).

Each refuge (GN box or fiberpot) was tested in four replicates, each within a simulated field environment box; each environment box was tested with an artificial host. An additional four environment boxes contained insects offered a blood meal but no refuges within to seek shelter. In a separate experiment, 3rd and 5th instars were introduced into environment boxes containing an artificial host with either GN box or fiberpots. The control environment box included fasted 3rd and 5th instars. The number and location of bugs inside and outside of the refuges were recorded for all environment boxes. Data were arcsin-transformed and compared by a one-way ANOVA. Significance was determined at levels of $p < 0.05$.

Recruitment abilities of different refuges

One small GN box and a single hole-punched fiberpot were placed in the same mosquito larval rearing tray (25.5 x 31 x 5.5 cm), lined with white paper, and observed over two days. Bugs tested per tray were 25 3rd instars, 10 4th instars, 10 5th instars, or 10 adults; instars were group tested by life stage; four replicates of each instar group were tested. The number of bugs inside and outside of each respective refuge were recorded

for each instar group. Data were transformed by arcsin and statistically analyzed by a t-test or one-way ANOVA. Significance was determined at $p < 0.05$.

Refuge recruitment and defecation patterns

A single fiberpot as used in previous experiments was placed within a plastic tub (29 x 34 x 13 cm) lined with white paper; ten chilled starved 5th instars were placed inside. One small GN box was placed within a mosquito larval rearing tray lined with white paper; 10 chilled starved 5th instars were also released inside. Five replicates of each type were performed. The number of bugs inside and outside of the refuge were recorded every day for one week and then, finally, 107 days after bug release. Defecation patterns were also observed.

A similar experiment was performed, except that: (1) all refuges were placed within a mosquito larval rearing tray and (2) bugs were left undisturbed. After 40 days, the number of bugs inside and outside the refuge were recorded and defecation patterns observed. Data were transformed by arcsin and analyzed by a t-test. Significance was determined to be when $p < 0.05$.

Comparison of refuge recruitment abilities

Four types of refuges were tested, as previously described (Format B). Two systems containing homogeneous groupings of each of the four refuges types were tested (2 x 4 = 8 boxes total) while three systems contained a mixture of the four refuge types. In each system, an artificial host was offered for 5 min. Nineteen refuges were placed in various strata of each system. Environment boxes containing one type of refuge only were

stratified into the floor, lower, middle, and upper walls and the ceiling region (3, 3, 3, 4 refuges respectively) while systems containing mixtures of refuge types were stratified into the floor, lower and upper wall, and ceiling regions (4, 4, 4, 4 refuges, respectively). Data were arcsin-transformed and compared by a one-way ANOVA. Significance was determined at levels of $p < 0.05$.

Comparison of potential attractants

Potential attractants were tested in a variety of ways (Table 23). Test materials included: Baker's yeast (emitting CO_2), *R. prolixus* feces, and ammonia gas. Cues were introduced to each port of the dual-port olfactometers (petri dish or Y-olfactometer) just prior to the introduction of chilled bugs. Five starved 4th instars were tested per petri dish while at least 10 bugs were tested per Y-olfactometer run. Olfactometers were permitted to operate overnight. In the morning, the number of bugs in each port was recorded. Petri dishes and cups were washed before each use. Data were arcsin-transformed and compared by a one-way ANOVA. Significance was determined at levels of $p < 0.05$.

Incorporation of a potential attractant within a refuge

Three test environment boxes, each containing 10 fiberpot refuges (3 small fiberpots glued together), were set up. Each environment box held 5 refuges contained 400 µl 240 µM NH_4OH while the other 5 contained 400 µl distilled water. Into each system, chilled bugs (15 3rd instars, 15 4th instars, and 10 5th instars) were released and permitted to roam through the system overnight. The next morning, the number and location of bugs found outside and inside of refuges was taken.

Another three environment boxes were set up in an identical manner as described above, except that insects also were provided with an artificial host. Data were arcsin-transformed and compared by a one-way ANOVA. Significance was determined at levels of $p < 0.05$.

Table 23. List of potential bug attractants and method of testing.

Substance	Y-olfactometer	Dual-port petri-dish	Passive-air model
Yeast[a]		X	
2.4×10^{1} µM NH_4OH[a]		X	
2.40×10^{2} µM NH_4OH[a]		X	
2.4×10^{3} µM NH_4OH[a]		X	
2.4×10^{4} µM NH_4OH[a]	X		
2.4×10^{5} µM NH_4OH[a]	X		
Colony feces[b]	X	X	X

[a]control = distilled water

[b]control = clean filter paper

Results

Comparison of two refuges by overnight dispersal of 3rd, 4th, and 5th instars

Data comparing refuges are in Tables 24-26. Bugs were recruited to both GN boxes and fiberpot refuges (Table 24): a significant number of bugs chose to enter either refuge instead of remaining outside ($p < 0.01$). There was no significant difference in recruitment ability between GN boxes or fiberpots, so the data were pooled. Fifth instars were significantly more likely to enter a refuge than third instars ($86.3 \pm 4.2\%$ vs. $55.5 \pm 7.2\%$) ($p = 0.0134$). Fifth instars also significantly preferred to rest in refuges located in the upper and lower wall regions rather than the ceiling ($p = 0.0010$), 4th instars significantly preferred the lower wall over the ceiling ($p = 0.0003$), but 3rd instars significantly preferred the lower wall first, followed by the upper wall and finally, by the ceiling ($p = 0.0009$) (Table 25).

Fourth and 5th instars released into control environment boxes which lacked refuges displayed a significant preference for residing in the ceiling region, over the floor and upper and lower walls ($p < 0.003$). Overall, when no refuges were provided, bugs significantly preferred the ceiling over the lower walls or floor ($p < 0.05$) (Table 26).

Overnight dispersal of nymphs: comparison of two refuges (3rd and 5th instars)

Comparison of refuge recruitment abilities with 3rd and 5th instars are in Tables 27-28. Though there was no significant difference in the percentage of 5th instars recruited by fiberpots or GN boxes, 3rd instars appeared to have been significantly more attracted to the fiberpots ($58.8 \pm 8.5\%$ vs. $30.8 \pm 7.8\%$) ($p = 0.0564$) (Table 27). Fifth

instars significantly preferred to migrate towards the upper walls and ceiling, rather than the lower walls ($p < 0.05$) (Table 28); they also exhibited a distinct preference for the upper regions of the wall versus the lower regions ($p < 0.05$) (Table 28).

There were no significant differences in the distribution of bugs inside refuges within either fiberpots or GN boxes for 5th instars, so the data were pooled. However, there was no significant difference between refuge locations in which 5th instars chose to reside, though most opted for the upper wall region (35.0 ± 10.5 % (upper wall) vs. 13.8 ± 6.0 % (ceiling) or 6.3 ± 3.2 % (lower wall)) (Table 28). A significantly higher proportion of 3rd instars in the upper regions rested in fiberpots than GN boxes, so the data could not be pooled ($p = 0.0304$) (Table 28). There were no significant differences of bug preferences for various strata within refuge types ($p > 0.05$).

Recruitment abilities of different refuges (choice experiments)

Fifth instars significantly preferred to seek refuge within a fiberpot than a GN box ($p = 0.0304$); no other life stages exhibited any significant preferences ($p > 0.05$) (Table 29). A significantly higher proportion of 5th instars were recruited into fiberpots than any other life stage ($p = 0.002$) with the GN boxes, fewer fifth instars were recruited than 3rd or 4th instars or adults ($p = 0.0571$). The vast majority of defecations passed by bugs resting in either a fiberpot or GN box were directly within and below the refuges, strongly suggesting that *R. prolixus* does not leave its refuge to defecate. However, the feces could still be acting as an attractant for other insects.

In another experiment in which 5th instars were offered refuge within either a GN box or fiberpot, there were no significant choice differences at either day 1 or day 8

($p = 0.1938$) (Table 30) though fiberpots appeared to be slightly more attractive ($60 \pm 13.8\%$ vs. $34 \pm 13.8\%$). However, the proportion of individuals seeking refuge within a GN box did significantly increase over the time interval ($p = 0.0114$) but not for those seeking refuge within a fiberpot ($p = 0.08$).

A similar experiment was performed, but with different time monitoring intervals (Table 31). The number of 5th instars recruited by fiberpots or GN boxes were not significantly different ($p > 0.05$). Though the number of bugs recruited by fiberpots did not significantly change from 12 hr to day 40 ($p > 0.05$), there was a significant increase for insects recruited by GN boxes ($p = 0.0026$). When the two groups were pooled, there was a significant increase in the number of bugs recruited overall from 12 hr to day 40 ($p = 0.0072$).

Recruitment abilities of different refuges (tested independently and simultaneously)

The proportion of bugs recruited within an environment box containing only fiberpots was significantly greater than those environment boxes containing any type of GN box (Table 32) ($p < 0.03$). When the dispersal of bugs was separated into strata, there were no significant differences except that black GN boxes recruited significantly fewer insects than all other refuge types in the upper wall region ($p < 0.05$), but significantly more insects in the ceiling region ($p < 0.05$).

Among environment boxes containing equal numbers of the four refuge types, the proportion of bugs seeking refuge in fiberpots was significantly higher than that of white GN boxes, cream GN boxes or black GN boxes (Table 33) ($p < 0.001$). Fiberpots recruited significantly higher proportions of 3rd and 5th instars, than any other refuge type

($p = 0.0098$ and 0.0001, respectively). Each refuge type had a similar proportion of recruited bugs within the varied strata ($p > 0.05$).

Attractant (yeast)

Five trials ($n = 25$ bugs) comparing the proportion of nymphs attracted to yeast (CO_2) versus distilled water indicated that the difference was statistically insignificant ($23.0 \pm 10.2\%$ vs. $57.0 \pm 16.6\%$, respectively) ($p > 0.05$) (data not shown). Observations in the laboratory indicated that the insects did not display any attraction towards the stimulus within 1 hr of the introduction of yeast.

Attractant (colony feces)

Bugs did not prefer to rest on filter paper containing bug feces ($p > 0.05$) in the passive-air model. However, there was a slight trend suggesting that the higher the bug density, the greater the tendency to aggregate or select bug feces (Table 34).

When the attraction of colony feces was tested within a dual port petri dish olfactometer overnight, no significant difference between bug attraction to feces or the control was observed ($50 \pm 22.4\%$ vs. $50 \pm 22.4\%$) ($p > 0.05$) (Table 35). Nymphs exposed to feces-coated and fresh filter paper for 1 hr in the Y-olfactometer significantly preferred the control over the feces ($79.2 \pm 12.5\%$ vs. $20.8 \pm 12.5\%$) ($p = 0.0275$) (Table 35). Fresh feces ranging in age from 3 hr to 21 hr elicited no significant reaction from *R. prolixus* when compared to clean filter paper ($16.7 \pm 10.9\%$ vs. $33.3 \pm 15.4\%$) ($p = 0.2959$) (Table 35). However, when feces approximately 12 hr old were placed in a chemical hood with a directional sash and an air flow, bugs were found to significantly

prefer the feces-coated paper over the control papers (59.3 ± 3.7% vs. 40.7 ± 3.7%) ($p = 0.0251$) (Table 35).

Attractant (ammonia gas)

Bugs did not display any significant attraction to 24 μM NH_4OH or 240 μM NH_4OH versus distilled water when tested overnight in a dual-port petri dish olfactometer or Y-olfactometer ($p > 0.05$) (Table 36).

Incorporation of a potential attractant into an artificial environment

Data comparing the recruitment of bugs into refuges containing a potential attractant are found in Tables 37-38. Proportions of fasted 3rd and 5th instar *R. prolixus* in refuges with or without ammonia did not significantly differ ($p > 0.05$). However, fasted 4th instars significantly preferred the refuges containing ammonia over those which did not ($p = 0.0533$) (Table 34).

In contrast, fed 3rd instars significantly preferred refuges lacking ammonia ($p = 0.0024$) (Table 37); there were no similar differences for 4th or 5th instars ($p > 0.05$). The refuge seeking behavior of 3rd instars significantly differed according to their hunger status: fed 3rd instars significantly preferred to seek refuges without ammonia when compared to their fasted counterparts ($p = 0.0274$) (Table 37).

Since there were no significant differences between the proportions of fasted or fed 4th instars seeking refuge, these data were pooled. Fourth instars significantly preferred refuges containing ammonia over those which did not ($p = 0.0034$) (Table 37).

Overall, fasted bugs significantly preferred to rest within refuges in the ceiling region rather than any other location within the environment box ($p = 0.0001$) (Table 38). Fed insects followed a similar pattern ($p = 0.0862$), in which the ceiling region was preferred ($p < 0.05$). In general, the proportion of fasted or fed bugs found in the ceiling was approximately 3-fold higher than that found in any other region. Fed bugs were less likely to seek a refuge than fasted bugs (67.1 ± 15.1 vs. $88.1 \pm 6.1\%$) but this difference was not significant and was probably due to the small sample size (6 boxes).

Table 24. Refuge recruitment of nymphs offered a small blood meal.

Refuge	Test Bugs	Percent Inside (± SEM)
GN boxes[a]	3rd instars (n = 40)	43.50 ± 4.7
	4th instars (n = 40)	72.50 ± 12.5
	5th instars (n = 40)	87.50 ± 6.3
	Combined (n = 120)	67.25 ± 6.3
Fiberpots[a]	3rd instars (n = 40)	67.50 ± 11.1
	4th instars (n = 40)	70.00 ± 7.1
	5th instars (n = 40)	85.00 ± 6.5
	Combined (n = 120)	74.00 ± 7.1

[a] = no statistical significance ($p > 0.05$)

Table 25. Distribution of nymphs resting within a refuge (a blood meal was offered on the floor of the environment box).

Test Insects	Percent of Bugs Within Refuges by Environment Box Strata (± SEM) Ceiling	Upper Wall	Lower Wall
3rd instars (n = 80)	5.0 ± 2.7^{c}	17.5 ± 5.3^{b}	31.3 ± 4.0^{a}
4th instars (n = 80)	6.3 ± 1.8^{e}	20.0 ± 5.0	46.3 ± 6.3^{d}
5th instars (n = 80)	10.0 ± 3.8^{g}	41.3 ± 5.2^{f}	35.0 ± 6.0^{f}

^{a,b,c} = statistically significant ($p < 0.05$)
^{d,e} = statistically significant ($p < 0.05$)
^{f,g} = statistically significant ($p < 0.05$)

Table 26. Distribution of nymphs in environment boxes without refuges (a blood meal was offered on the floor of the environment box).

	Percent of Bugs Inside (± SEM)		
Strata	3rd instars (n = 80)	4th instars (n = 80)	5th instars (n = 80)
Ceiling	59.0 ± 14.7[a]	57.5 ± 8.8[a]	67.5 ± 11.1[a]
Upper wall	12.8 ± 6.3[b]	27.8 ± 10.9[b]	30.5 ± 10.2[b]
Lower wall	12.8 ± 2.4[b]	10.3 ± 3.8[b]	0.0 ± 0.0[b]
Floor	15.5 ± 6.6[b]	4.5 ± 4.5[b]	0.0 ± 0.0[b]

[a,b] = statistically significant ($p < 0.05$)

Table 27. Recruitment of nymphs by refuge types (a blood meal was offered on the floor of the environment box).

Refuge	Test Insects	Percent Inside (± SEM)
GN boxes	3rd instars (n = 85)	30.8 ± 7.8[a]
	5th instars (n = 30)	66.7 ± 6.9
	Combined (n = 115)	37.0 ± 11.2
Fiberpots	3rd instars (n = 89)	58.8 ± 8.6[b]
	5th instars (n = 40)	80.0 ± 9.0
	Combined (n = 129)	66.0 ± 9.6

[a,b] = statistically significant (p = 0.564)

Table 28. Distribution of nymphs resting within a refuge (a blood meal was offered on the floor of the environment box).

		Percent of Bugs Within Refuges by Environment Box Strata (± SEM)		
Test Insects	Refuge	Ceiling	Upper Wall	Lower Wall
3rd instars (n = 174)	GN boxes	13.7 ± 5.0	11.3 ± 2.5[e]	13.0 ± 7.2
	Fiberpots	20.4 ± 6.0	27.8 ± 5.1[d]	17.5 ± 5.2
5th instars (n = 70)[a]	Pooled	13.8 ± 5.0[b]	35.0 ± 10.5[b]	6.3 ± 3.2[c]

[a] = GN boxes and fiberpot refuges pooled
[b,c] = statistically significant ($p < 0.05$)
[d,e] = statistically significant ($p < 0.05$)

Table 29. Recruitment of fasted nymphs offered a choice between two refuge types.

	Percent Inside (± SEM)	
Test Insects	GN Boxes	Fiberpots
3rd instars (n = 100)	37.0 ± 6.2[c]	25.0 ± 2.5
4th instars (n = 40)	27.5 ± 4.8[c]	47.5 ± 12.5
5th instars (n = 40)	2.5 ± 2.5[a,d]	92.5 ± 2.5[b]
Adults (n = 40)	40.0 ± 15.8[c]	30.0 ± 10.8

[a,b] = statistically significant ($p < 0.05$)

[c,d] = statistically significant ($p < 0.05$)

Table 30. Recruitment of 5th instars (n = 50) by either Gomez-Nunez boxes or fiberpots (bugs were not permitted a choice between refuges).

	Percent Inside (± SEM)	
Observation Day	Fiberpots (n = 50)	GN Box (n = 50)
Day 1	60.0 ± 13.8	34.0 ± 13.8[a]
Day 8	86.0 ± 0.1	86.0 ± 0.1[b]

[a,b] = statistically significant ($p < 0.05$)

Table 31. Recruitment of 5th instars by various refuge types (day 1 and day 40).

	Percent Inside (± SEM)	
Observation Day	Fiberpots (n = 50)	GN Box (n = 50)
Day 1	62.0 ± 10.7	34.0 ± 8.7[a]
Day 40	79.9 ± 6.7	85.6 ± 6.7[b]

[a,b] = statistically significant ($p < 0.05$)

Table 32. Recruitment of nymphs within environment boxes containing only one refuge type.

	Percent of Bugs Inside by Refuge Type (± SEM)			
Test Insects	White	Cream	Black	Fiberpot
3rd instars	53.5 ± 26.5 (n = 21)	50.0 ± 20.0 (n = 20)	48.0 ± 19.0 (n = 16)	83.5 ± 17.0 (n = 7)
4th instars	30.0 ± 30.0 (n = 18)	35.0 ± 15.0 (n = 19)	50.5 ± 20.5 (n = 17)	69.0 ± 2.0 (n = 13)
5th instars	55.0 ± 15.0 (n = 21)	62.5 ± 17.5 (n = 21)	43.0 ± 7.0 (n = 17)	83.5 ± 17.5 (n = 23)
Combined	45.0 ± 18.0 (n = 60)[a]	32.0 ± 1.0 (n = 60)[a]	27.0 ± 0.0 (n = 50)[a]	78.0 ± 11.0 (n = 43)[b]

[a,b] = statistically significant ($p < 0.05$)

Table 33. Recruitment of nymphs within environment boxes containing equal amounts of various refuge types.

	Percent of Bugs Recruited by Refuge Type (± SEM)			
Test Insects	White	Cream	Black	Fiberpot
3rd instars	9.0 ± 4.5	26.7 ± 7.3	8.3 ± 8.3	56.7 ± 10.7
4th instars	11.0 ± 11.0	27.7 ± 5.3	11.0 ± 11.0	50.0 ± 25.4
5th instars	4.3 ± 4.3	8.7 ± 4.3	18.7 ± 3.5	69.3 ± 6.3
Combined	4.0 ± 1.0[a]	13.3 ± 3.7[a]	9.0 ± 1.0[a]	45.3 ± 1.5[b]

[a,b] = statistically significant ($p < 0.05$)

Table 34. Percentage of bugs resting on either feces-coated or clean filter paper (passive-air model).

	Percent of Insects (± SEM)		
Filter Paper Type	1 Bug	5 Bugs	10 Bugs
Feces	40 ± 12.6	52 ± 7.4	59 ± 9.7
Control	48 ± 10.2	43 ± 9.8	41 ± 9.7

Table 35. Percentage of bugs preferring either feces-coated or clean filter paper (olfactometers).

		Percent of Bugs (± SEM)		
Test Type	Age of Feces	Feces	Control	p-value
Dual-port olfactometer	1 yr	50.0 ± 22.4	50.0 ± 22.4	1.0000
Y-olfactometer	1 yr	20.1 + 12.5	62.5 + 14.2	0.0275
Y-olfactometer	3 - 21 hr	16.7 + 10.9	33.3 + 15.4	0.2959
Y-olfactometer	12 hr	59.3 + 3.7	40.7 + 3.7	0.0251

Table 36. Percentage of nymphs attracted to various concentrations of ammonium hydroxide.

NH_4OH (μM)	Percent Attracted to NH_4OH (± SEM)	Percent Attracted to Distilled Water (± SEM)	p-value
24 μM[a]	50.0 ± 22.4	30.0 ± 20.0	0.5212
240 μM[a]	44.1 ± 8.6	35.1 ± 8.1	0.3933
2,400 μM[a]	42.7 ± 14.8	47.3 ± 2.0	0.7775
24,000 μM[b]	37.5 ± 37.5	62.5 ± 37.5	0.5972
240,000 μM[b]	0.0 ± 0.0	100 ± 0.0	N/A[c]

[a] = tested overnight in a dual-port petri dish olfactometer
[b] = tested overnight in a Y-olfactometer
[c] = sample size is too small for statistical analysis but data suggests repellency effect

Table 37. Percentage of nymphs seeking refuge in an environment box containing refuges with or without 400 µl of 240 µM NH_4OH.

		Percent Inside (± SEM)		
Group	Status	3rd instars	4th instars	5th instars
Control	Fasted	47.5 ± 5.5	44.3 ± 3.0[a]	58.5 ± 1.5
Experimental	Fasted	52.4 ± 5.5	55.7 ± 3.0[b]	48.1 ± 6.0
Control	Fed	70.8 ± 4.2[c]	44.3 ± 3.7	48.6 ± 13.2
Experimental	Fed	29.2 ± 4.2[d]	55.7 ± 3.7	51.4 ± 13.2

[a,b] = statistically significant ($p < 0.05$)
[c,d] = statistically significant ($p < 0.05$)

Table 38. Dispersal of nymphs within an environment box containing refuges with or without 400 µl of 240 µM NH_4OH.

	Percent Inside (± SEM)				
Status	Floor	Wall1[a]	Wall2[a]	Wall3[a]	Ceiling
Fasted	10.9 ± 3.3[b]	14.1 ± 5.3[b]	13.6 ± 5.0[b]	15.3 ± 3.5[b]	47.0 ± 5.8[c]
Fed	16.8 ± 5.7	13.1 ± 4.8	12.5 ± 6.3	12.1 ± 6.8	45.5 + 11.7

[a] = correspond to the three lateral walls of the environment box
[b,c] = statistically significant ($p < 0.05$)

Discussion

Recruitment of bugs into refuges

Nymphal *R. prolixus* seem to maintain their position, once they find a satisfactory refuge. Until they do, *R. prolixus* seem to continue traveling upward. This is supported by the fact that in the absence of refuges, bugs prefer to rest on the ceiling.

For the most part, 4th and 5th instars appear to seek a refuge more readily than 3rd instars. Perhaps this is due to feeding frequency since older instars can tolerate extended periods of starvation. The tendency to seek a refuge also appears to have a physiological basis; insects offered a small meal were found to seek a refuge less frequently than those that remained fasted. My data also suggest that there may be some behavioral differences between instars, which would probably affect their attraction to material substances located in various strata. Fifth instars were more likely to seek a refuge than third instars. After ecdysis, adults may seek a refuge to find a mate and to lay eggs. Smaller instars may be less conspicuous and thus, have less need to seek a refuge.

Overall, fiberpot refuges appear to be more attractive to *R. prolixus* nymphs than paper GN boxes. Fiberpot substrate is rough and the interior is well-protected from light, though the bugs were found resting on all parts of the refuge. Fiberpot material is a poor indicator of bug defecation activity since it is very dark, making it difficult to identify feces. To determine whether or not the bias towards fiberpots was due to its color contrast against Sculptamold, which dries white, one experiment tested three colors of GN boxes with two different substrates, smooth versus rough. Though considered to be most similar to the fiberpot, the black construction paper GN boxes did not recruit more bugs than cream construction GN boxes. Construction paper, though not as rough as fiberpot

material, is probably rougher than cardboard, the usual material of GN boxes. Kissing bugs orient by thigmotaxis and prefer substrates which lend themselves readily to vibrations and good footholds.

According to these laboratory experiments, fiberpots appear to hold potential as a good bug refuge and therefore, as a possible population monitoring device. Recruitment levels within the artificial environments were consistently high. Fiberpots are mass-produced at low cost. At a local nursery, the experimental fiberpots sold for $0.05 each. Bulk or wholesale purchasing would reduce this price substantially.

Attractants

Despite many experiments, none of the substances tested appeared to act as a kissing bug attractant, except for some ammonia concentrations in some trials. High concentrations of ammonia may even repel kissing bugs. However, fasted 4th instars did significantly prefer refuges containing ammonia. In contrast, fed 3rd instars significantly preferred refuges lacking ammonia. In general, it seems doubtful that there is a single component that is as attractive to kissing bugs as live hosts. Though my data indicate ammonia gas has potential as a bug attractants, there remains the strong possibility that only fasted bugs will be attractant.

Defecation patterns

According to the literature, *T. infestans* reportedly leaves its refuge to defecate outside. However, observations of the fecal patterns produced by *R. prolixus* within Gomez-Nunez boxes and fiberpots suggest that these bugs do not do this. An

overwhelming majority of fecal drops were located directly below the refuge (fiberpot design) or on paper towels (contained within the paper GN boxes). The presence of feces within the refuge could act as a potential attractant.

Simulated field environment box

This novel artificial system demonstrates promise as a possible artificial field environment to test the efficacy of new attractants or traps. Each system, being constructed out of Styrofoam, was extremely light, inexpensive, mobile and yet durable. A pre-determined number of bugs can be introduced into the system and be recaptured. Refuges could be readily attached to the Sculptamold substrate at any position and in addition, the substrate itself could be rinsed free of any dirt or feces. Sculptamold also resembles a mud-like substrate; if applied by hand, the substrate can be created to be as rough or smooth as desired. Touch-up repairs dry overnight. Kissing bugs easily walked about the Sculptamold surface, even on the ceiling. In the absence of suitable refuges, the majority of *R. prolixus* migrated to the upper wall and ceiling regions. In nature, *R. prolixus* prefer huts with thatched roofs, where they generally reside.

A few minor disadvantages of the system were noted. Though covering the front of the environment box with Saran Wrap provided a convenient mode to introduce bugs and refuges, the plastic was susceptible to temperature changes and sometimes stretched, even though it was secured by thumbtacks. Unfortunately, this led to the loss of several bugs. Perhaps a more suitable cover could be used; however, if the material is too heavy, the entire box may topple over. Also, it was impossible to regulate the humidity levels

within the box. Experiments of longer duration on the order of several weeks may result in increased mortality.

My data suggest that there are several important differences between *R. prolixus* and *T. infestans*. Substances that reportedly attract *T. infestans* may have little effect on *R. prolixus*. *Triatoma infestans* may innately prefer to inhabit different strata of a room than *R. prolixus*, perhaps due mainly to differences in thigmotaxis and oviposition and defecation activity. *Rhodnius prolixus* also defecates sooner than *T. infestans*, though it needs to feed less frequently. These two factors alone may dictate the proximity to a host that each species prefers to hide. In addition, the hunger status of *R. prolixus* affects whether it seeks a refuge or not. The location and type of population monitoring device must consider several behavior and physiology parameters in order to increase the chances of detection. Without detection and identification of bug infestations, proper control measures cannot be implemented.

CHAPTER 7

SUMMARY OF CONCLUSIONS

My research produced the following conclusions:

1. *Trans*-sialidase does not appear to affect *R. prolixus* mortality, nymphal development or adult fecundity.

2. Simultaneous ingestion of *Trypanosoma* or recombinant TS types with *T. cruzi* does not consistently affect the parasite load in *R. prolixus* after 33-47 days incubation. Ranges of enzyme tested were: 2,337-955,700 cpm/ml blood (*Trypanosoma* TS), 550 - 5828 cpm/ml blood (19y rTS) and 1469-5834 cpm/ml blood (rTS2000).

3. After 24 hr, *Trypanosoma* TS activity was detected in the gut extracts of *R. prolixus*. TS levels produced by *T. cruzi*-infected controls were much less than those of bugs ingesting exogenous TS. However, bugs ingesting exogenous TS with *T. cruzi* parasites had comparable TS activity levels to bugs ingesting only TS within an uninfected blood meal.

4. TS (19y rTS) remains catalytically active and detectable in the gut of *R. prolixus* nymphs for 10-12 days.

5. Ingestion of mAb TCN-2 with *T. cruzi*-infected blood meals resulted in increased parasite loads in *R. prolixus* when compared to infected controls. Through a series of successive experiments, the optimal concentration of monoclonal antibody concentration was determined to be 1.0 μg mAb/ml blood.

6. The addition of polyclonal antibody (1.0 μg/ml blood), specific for the tandem repeats in the carboxyl-terminus of the TS enzyme, also significantly increased *T. cruzi* parasite loads in *R. prolixus*.

7. Bugs ingesting purified subpopulations of TS^- parasites had significantly higher parasite loads after 33 days than bugs ingesting TS^+ or unfractionated *T. cruzi* parasites.

8. Ingestion of TS^- parasite subpopulations incubated with *Trypanosoma* (6,000-408,500 total cpm) or recombinant TS types (600-38,000 total cpm) for 30 min significantly decreased parasite loads to levels intermediate between those of bugs ingesting TS^- parasites and those ingesting TS^+ parasites.

9. The addition of TS monoclonal antibody (1.0 μg/ml blood) to fractionated Silvio *T. cruzi* subpopulations significantly increased the parasite loads of TS^+ and unfractionated parasites, but not TS^- parasites.

10. Maximal parasite loads appear to occur at approximately 30 days post-ingestion.

11. Ingestion of fractionated subpopulations of two other *T. cruzi* strains (Tulahuen and MV-13) follow the same trend of parasite subpopulation development as that of the Silvio strain. Bugs ingesting TS^- parasite subpopulations have higher parasite loads than those ingesting TS^+ or unfractionated parasites.

12. These experiments suggest that trypomastigote populations are polymorphic, in order to increase the parasite's chances for invading either the invertebrate or mammalian system. TS^+ parasites are more adapted towards surviving and invading mammalian cells while TS^- parasites appear to thrive more successfully in the insect host than TS^+ parasites.

13. Among those insects failing to molt, those which were starved for longer periods (5-6 months) required significantly less time to feed than those insects fasted for shorter periods (2-3 months).

14. The number of probing attempts significantly increased as the fasting period increased. Bugs that failed to molt to the next instar were more likely to probe repeatedly than those that successfully molted.

15. Bugs that fasted for longer periods of time (5-6 months) defecated significantly earlier than those that fasted for shorter periods (2-3 months). Defecation occurred earlier for bugs ingesting two versus one blood meals.

16. Infected bugs, fed through an artificial membrane and starved for at least 2-6 months, defecated significantly more often up to 5 min post-feeding than their uninfected counterparts. However, a reversed trend was observed with bugs fed on a live host and starved for shorter periods (2-5 wk): infected bugs defecated significantly less frequently than uninfected bugs.

17. The more fasted bugs were, the less blood they ingested, regardless of whether or not they successfully molted.

18. Defecation indices increase as levels of starvation decrease.

19. Based on defecation indices, 5th instars appear to have the highest vector potential, followed by adult females, 4th instars, and then adult males.

20. As bugs become progressively more starved, their response levels to host stimuli are decreased. However, infected bugs starved for 4-6 months responded more quickly to a human stimulus than uninfected bugs.

21. *Rhodnius prolixus* nymphs prefer to rest on the ceiling and upper regions of the artificial environment box.

22. Fourth and 5th instars appear to seek refuge more frequently than 3rd instars. This may be due to difference in life stage (leading up to potential mate-seeking) or perhaps a difference in feeding frequency (increased abilities to resist starvation).

23. Bugs offered a small blood meal were less likely to seek refuge than fasted bugs.

24. Fasted fourth instars significantly preferred to rest inside of refuges containing 240 μM ammonia than those that lacked the substance.

25. Fed third instars significantly preferred to rest within control refuges rather than those that contained ammonia.

26. The artificial styrofoam environment holds potential as a method in which to test, in a controlled manner, future bug attractants. The pre-determined number of bugs can be observed and the number or type of refuges can be manipulated with ease. Each artificial system is re-usable and maintenance costs are low.

Millions of people are still at risk of Chagas' disease in Latin America. However, efforts to develop a parasitological cure to control the parasite within the vector are many years from fruition.

In mammals, evidence that *trans*-sialidase plays a role in infection of cells continues to grow. Researchers know that the enzyme persists and remains active throughout the course of infection but the reasons remain unknown. Future studies should focus on the two morphological forms of *T. cruzi* trypomastigotes and examine if other phenotypic differences exist. If a substance could be withheld from the trypomastigotes, rendering them incapable of invading mammalian cells, then parasitemia could be significantly reduced. I suspect that phenotypic expression of TS may be linked to the expression of other proteins, which facilitate invasion of cells.

In evolutionary terms, there appears to have been little to no pressure for *T. cruzi* to express TS while inside the invertebrate vector, since triatomids do not produce sialic acid and sialic acids are found in plentiful amounts within a blood meal. Selection for sialylated parasites probably only became necessary once the triatomids began interacting with mammals. My data strongly suggest, however, that parasites that do not phenotypically express TS (TS^-) are more adapted towards surviving in the insect vector, while TS^+ parasites are more capable of invading mammalian tissues.

The relationship between triatomids and humans probably has not existed long enough to encounter high selection pressure towards bugs that defecate more quickly. Mammals acting as reservoirs of *T. cruzi* probably acquire the infection by ingesting infected bugs. Humans are more likely to scratch the infective feces into their skin or mucosal membranes, becoming infected in this manner instead. However, if humans were the primary reservoir of *T. cruzi* and triatomids displayed obvious preferences for feeding on man, then one might expect selection pressure for a strain of *T. cruzi* that increased the defecation activity of the bug. At this time, though, it appears that the presence of the

parasite subtly affects the hindgut of the bug as to reduce defecation behavior. Parasite presence might affect the ability of the Malpighian tubules to release excess water or negate the impact of the diuretic hormone. Preliminary histological studies with transmission electron microscopy do not suggest that the parasite causes any histological damage to the gut, but the fact that parasites prefer to attach to the rectal ampulla suggests that there is something about that location which appeals to or attracts the parasite.

The most promising method of controlling Chagas' is through vector control. However, behavioral studies have been sadly neglected so generalizations about bug behavior are made across species. *Rhodnius prolixus* displays a preference towards climbing towards higher regions of the wall and ceiling, as well as preferring certain kinds of refuge. Instar age and the physiological status of hunger also appears to affect the tendency to seek a refuge. When attempting to monitor bug infestations, especially at low densities, exploiting the behavioral tendencies of the targeted insect species would seem a more successful technique than merely placing a cardboard box near a bed. My studies suggest that successful bug collection by the passive means of a refuge box would depend heavily on several factors, all affected by the strategic location of the box:
(1) preferred resting substrates, (2) level of fastedness, (3) instar age, (4) availability of competing refuges, and (5) proximity to a blood meal. The proposed simulated field environment box holds potential as a method in which to observe bug behavior within the laboratory. Bugs are allowed a wide range of movement and the situation provides realism than an olfactometer.

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